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NONTREPONEMAL AND TREPONEMAL TESTS FOR SYPHILIS*

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Tests for syphilis in current use are of two types—nontreponemal and treponemal. The older nontreponemal tests have been modified and new tests have been developed that use cardiolipin antigen. Treponemal tests were originally performed only in research and reference laboratories. Simpler test procedures using treponemal antigens have since been described that are practical for use in public health and local laboratories. The Serology Evaluation and Research Assembly (SERA) Study[19] conducted by the United States Public Health Service in 1956-57 evaluates both types of tests. Definite trends that have developed during the past decade, and current knowledge regarding serologic tests used in public health and local laboratories in this country are reviewed.

Manual of Serologic Tests for Syphilis

Beginning in 1939, the Venereal Disease Research Laboratory of the United States Public Health Service, in collaboration with the author-serologists, has been responsible for preparing manuals of Serologic Tests for Syphilis that are published periodically. The manuals contain the latest revision of existing technics and include new technics that have been published and are in use in the United States. Tests that are not being used to an appreciable extent are deleted.

The contents of the three Manuals of Serologic Tests for Syphilis[8,17,18] exemplify the developments in syphilis serology during the ten year period 1949-1959.

CHART I

Cardiolipin antigens have replaced lipoidal antigens in the older nontreponemal tests for syphilis, e.g., Hinton, Kline, Kolmer and Mazzini tests. The Kahn test may be performed with either lipoidal or cardiolipin antigen. The VDRL tests for syphilis were developed to make use of cardiolipin and lecithin as antigen components.

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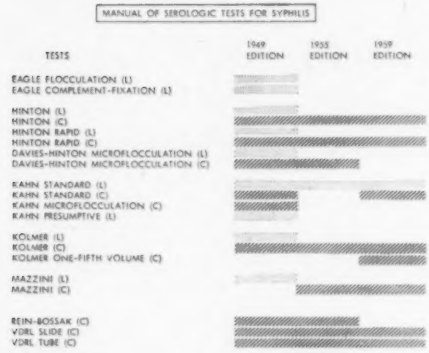
CHART 1



The 1959 Manual of Serologic Tests for Syphilis[18] includes the technic for the Rapid Plasma reagin (RPR) test published by Portnoy, Garson and Smith[14] (1957). This is the first of a group of rapid tests for syphilis designed as "screening" tests. Blood for the RPR test is collected into tubes containing an anticoagulant, preferably potassium sequestrene if the blood is to be used for serologic testing only. The blood specimen is centrifuged to separate the plasma from the blood cells. Plasma for the test is pipetted from the collecting tube and examined without heating. The RPR antigen suspension is prepared by resuspending sedimented VDRL antigen emulsion particles in 10 per cent choline chloride solution. For the test proper, three drops of plasma from each specimen is placed in a concavity of a Boerner slide, and one drop of RPR antigen suspension is added to each test. Tests are then rotated for four minutes at 180 r.p.m. on a mechanical rotator circumscribing a circle three-fourth inch in diameter. Test results are read immediately after rotation with a microscope at 100 X magnification. The RPR test has been used successfully in field operations on the U. S. Border for testing Mexican migrant laborers entering this country where it was desirable to complete the testing of large groups rapidly and economically. An evaluation of the RPR test in such a field operation was published by Simpson *et al.*[16] (1959). The test has also been used in general population surveys using blood specimens collected for diabetic screening tests.

Two modifications of the RPR test have been published since the text of the 1959 Manual of Serologic Tests for Syphilis was completed. These

are the Unheated Serum Reagin (USR) test[19, pp. 92-93] and the Rapid Plasma Reagin (RPR) test with unheated serum.[13] Reports[2,12] have been published comparing the results obtained with these tests with those of the VDRL Slide test.

The Plasmacrit (PCT) test, devised by Andujar and Mazurek (1959) is essentially a "micro-RPR" test which utilizes the plasma remaining in a micro-hematocrit tube after the determination of the packed cell volume. The test, performed with capillary blood obtained by fingertip puncture, eliminates the necessity of obtaining blood by venipuncture. An evaluation by the Venereal Disease Research Laboratory[10] shows that the PCT test was more reactive than the VDRL Slide test on 599 presumably nonsyphilitic individuals and on specimens from 109 patients having syphilis or a history of treatment for syphilis. As a screening procedure, the test seems best suited for use in blood banks, hospitals and private laboratories, and possibly in mass surveys.

Treponemal antigen tests are included in the 1959 Manual of Serologic Tests for Syphilis. Three of these tests, the Treponema Pallidum Immobilization (TPI) Treponema Pallidum Complement Fixation (TPCF) and the Fluorescent Treponemal Antibody (FTA) tests employ the virulent *Treponema pallidum* as an antigen. The fourth treponemal test is a small volume Kolmer test using reiter protein antigen extracted from the Reiter treponeme (KRP). In the United States the TPI test is performed in reference laboratories of the Army, Navy, Public Health Service, and in several of the larger State health department and university laboratories. The test is technically difficult, time-consuming, and expensive to maintain in operation. It has achieved considerable stature as a test which may be used to differentiate the reactions due to syphilis from biologic false positive reactions.

The original TPCF test using an extract of virulent *Treponema pallidum* as antigen was published by Portnoy and Magnuson[15] (1956). The third modification of this test using small volumes of reagents and published[12] (1958) as the "tpcf-50" test for syphilis is the method recommended by the author in the 1959 Manual of Serologic Tests for Syphilis .This test is less expensive than the original test, since it uses one-eighth the amount of antigen, and it is completed in a single day. A comparative study[3] made of the three TPCF tests and the TPI test has been reported by the Venereal Disease Research Laboratory.

The FTA test was published by Deacon, Falcone and Harris[7] (1957). A suspension of virulent *Treponema pallidum* in the basal medium recommended for the TPI test is used as the test antigen. This antigen may be stored in the liquid state or may be preserved by freeze-drying for long periods of time. The test utilizes the indirect fluorescent antibody technic to detect treponemal antibodies in human test serum. A darkfield fluorescent microscope assembly is used for the microscopic reading of test results. The FTA test technic is simple, and reagents are relatively inexpensive. The microscope and ultraviolet light source represent a considerable investment.

The one-fifth volume Kolmer test with Reiter protein antigen (KRP)[4] is the most practical and economical of the treponemal test procedures for use in public health and clinical laboratories. The test uses a protein antigen

extract from the avirulent Reiter treponema[20] prepared in general accordance with the method developed by D'Alessandro and Dardanoni[6] (1953).

National and Intrastate Serologic Evaluation Studies

The Venereal Disease Research Laboratory conducts an annual USPHS Serologic Evaluation Study in which all the State health department laboratories in this country, and several foreign countries, participate. A comparison of the tests entered in this evaluation in different years shows the tests that are in use in major public health laboratories.

TABLE I

Table I indicates the number of laboratories performing each of the tests entered in the serology evaluation study in fiscal years 1950, 1955 and 1960.

TABLE I

Tests Performed by Laboratories in USPHS Serologic Evaluation Studies

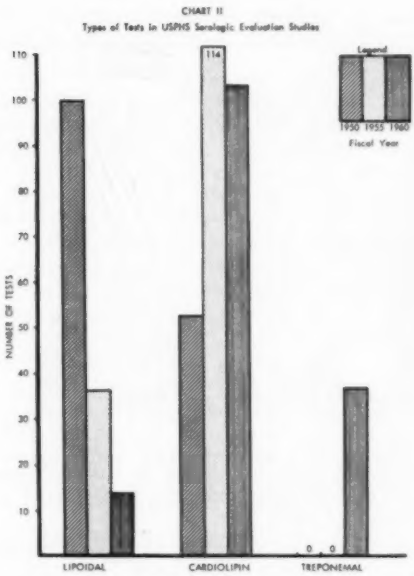
Test	Fiscal Year		
	1950	1955	1960
VDRL Slide	35	46	49
Kolmer			
Lipoidal	30	4	1
Cardiolipin	2	34	26
Kolmer 1/5 Volume			
Cardiolipin	0	0	3
Reiter Protein	0	0	34
Kahn			
Lipoidal	28	21	11
Cardiolipin	0	0	0
Mazzini			
Lipoidal	15	4	0
Cardiolipin	0	6	6
Kline			
Lipoidal	2	1	0
Cardiolipin (NL)*	10	9	10
Cardiolipin (SL)**	0	0	1
Hinton			
Lipoidal	7	0	0
Cardiolipin	0	8	7
VDRL Tube	4	4	2
Eagle Flocculation			
Lipoidal	4	1	0
Fluorescent Treponemal Antibody	0	0	3
Miscellaneous	15	12	3

* Natural Lecithin.
** Synthetic Lecithin.

CHART II

Chart II summarizes the data presented in Table I and indicates the number of tests performed in these three surveys using lipoidal, cardiolipin and treponemal antigens.

Many State health department laboratories in the United States conduct intrastate serology evaluation studies in connection with their



programs for the approval of laboratories to perform tests for syphilis required by law. The number of laboratories participating in these studies during the past ten-year period has almost doubled. Data from four intrastate studies conducted in 1950, 1955, and 1959 was collected. Chart III indicates the percentage of the total number of laboratories included in these studies that performed the Kahn Standard, Kolmer Complement-Fixation, Kline Standard, Hinton, Mazzini and VDRL Slide tests for syphilis.

CHART III

Serology Evaluation and Research Assembly (SERA) Study

The increasing number of serologic tests for syphilis, both non-treponemal and treponemal, and modification of existing technics, resulted in a recommendation by the National Advisory Serology Council in 1955 that the Public Health Service conduct a study to evaluate the sensitivity and specificity of tests using lipoidal and cardiolipin antigens and the new group of tests using treponemal antigens. This study, known as the Serology Evaluation and Research Assembly (SERA) Study, was conducted in 1956-57. Thirty-eight tests for syphilis were performed on approximately 1300 specimens in ten diagnostic categories by 14 participating laboratories. The reports and test results were forwarded to the Venereal Disease Branch, Communicable Disease Center, Atlanta, Georgia, the tabulating center. A complete report of the

CHART III
Tests Performed in Intrastate Serologic Evaluation Studies

results of this study has been published as Public Health Service Publication No. 650.[19] The following excerpts summarize the principal conclusions of the Public Health Service.

1. . . . "The purpose of serologic testing is to detect the presence or absence of syphilitic 'antibodies' at a selected reactivity level. Good sensitivity with concomitant good specificity must be the guideline of an adequate test since an individual test will be applied in ordinary use to a patient of undetermined status, syphilitic or nonsyphilitic. . . . It is suggested by this study that increases in sensitivity in both treponemal and nontreponemal tests beyond certain limits leads to a loss of specificity.
2. The results in various categories in this study also demonstrate the fallacy of arbitrarily accepting the TPI or any test identifying so-called specific antibodies as having absolute sensitivity or specificity. . . .
3. As a new procedure, the combined sensitivity and specificity of the various Reiter protein antigen tests in this study as well as the reproductibility of results in different laboratories indicate the value of routine performance of a form of this test as a valuable serologic aid to diagnosis.
4. The ratings of sensitivity and specificity based on SERA data can be interpreted as supporting the continued widespread use of a cardiolipin or a lipoidal test of good sensitivity and good specificity, and the trial supplementary use of a Reiter protein antigen test, either (1) routinely on all specimens or (2) as a check on all reactive specimens or (3) in questionable Biologic false positive (BFP) cases.
5. Interpretation of the results in both treponemal and nontreponemal tests must be done in the light of clinical and historical findings. . . . Performance of 38 tests on any serum points up the fact that the individual serologic pattern is a complex of variables of the disease

and patient, e.g., stage of syphilis, specific or incidental treatment, antibody responses of the individual, antigen and techniques used."

Discussion

Nontreponemal tests for syphilis utilize lipoidal and cardiolipin antigens and detect an antibody known as *reagin* which develops as the result of syphilitic infections. Older tests for syphilis used the lipoidal-type antigen—a crude extract of animal tissue—particularly beef heart muscle. Pangborn[11] (1941) isolated cardiolipin, the substance responsible for the reactivity of lipoidal antigens in tests for syphilis. This resulted in the development of antigens composed of cardiolipin, purified lecithin, and cholesterol known as cardiolipin antigens which proved to be more reproducible than the lipoidal antigens. Tests performed with cardiolipin antigens demonstrated an increased specificity and sensitivity. By 1955, the Kahn Standard test was the only widely used test in this country still employing a lipoidal antigen.

The shift from test technics employing lipoidal antigens to those with cardiolipin antigens is shown in Chart I, listing the tests included in the 1949, 1955 and 1959 Manuals of Serologic Tests for Syphilis; in Table I and Chart II showing tests performed in USPHS Serologic Evaluation Studies; and in Chart III, indicating the tests performed by laboratories participating in intrastate evaluation studies in four states.

A screening procedure, the Rapid Plasma Reagin (RPR) test,[14] is included in the 1959 Manual of Serologic Tests for Syphilis. The Plasmacrit (PCT) test[1] and the RPR tests[13,19 (pp. 92-93)] with unheated serum using the same antigen suspension have recently been published. These tests are supersensitive and when a reactive or weakly reactive result is obtained, the test-authors and the National Advisory Serology Council in its report of a meeting held in 1959 suggest that it is advisable to perform a "standard" test for syphilis on serum from the patients before issuing a report which might be used as an aid in the diagnosis or treatment of syphilis.

The four treponemal tests included in the 1959 Manual of Serologic Tests for Syphilis (FTA, TPCF, TPI and KRP) represent the tests that are currently in use or show promise of being used in public health and clinical laboratories. The TPI test was the first of the group of treponemal tests employing virulent *Treponema pallidum* as an antigen. A national TPI testing service has been available from the Venereal Disease Research Laboratory since January 1955 to private physicians who may refer specimens on patients who meet certain criteria through their respective state health departmental laboratories. The test is not practical for use in routine laboratories. The FTA test is being used experimentally in a number of public health and university laboratories. The TPCF test has certain limitations because the antigen must be stored at —20°C. and is rather expensive. The KRP test can be performed in any laboratory that has the facilities to perform complement-fixation tests. It will be noted from Table I that over 30 laboratories are performing this test in the USPHS Serologic Evaluation Study for Fiscal Year 1960. Chart III does not indicate that treponemal tests are in use in local laboratories. This is possibly due to the fact that this type of test would not

ordinarily be used for premarital and prenatal examinations. A number of state laboratories not included in the data on this chart are evaluating or contemplating the evaluation of test performance of the KRP test. Instruction in test performance of the KRP test has been included in serology workshops sponsored by state health departments during the past two years. In addition to the results obtained in the SERA Study, published results of comparative testing with the VDRL Slide, the TPI and KRP tests are summarized by Brown and Bunch[5] (1959) as follows: "the KRP test becomes reactive almost as rapidly as the VDRL Slide test following infection, maintains reactivity longer than the VDRL Slide test, but, given time, will revert to negative in the same proportion as the VDRL Slide test; by comparison it roughly parallels the TPI test in the nonsyphilitic, including biologic false positive, categories." Harris, Bossak and Wallace[9] (1959) compared the results obtained with the VDRL Slide, KRP and TPI tests on specimens submitted for the TPI testing service of the Veneral Disease Research Laboratory to physicians in the continental United States, Alaska, Hawaii, Puerto Rico, and the Virgin Islands, and concluded that if used only on selected patients, the VDRL Slide and KRP tests might serve effectively in the serological preselection of serums for the TPI test.

Summary

The past decade—1950 to 1960—has seen a definite shift in the use of nontreponemal tests for syphilis employing lipoidal antigens to those with cardiolipin antigens. The number of treponemal tests has increased and four are included for the first time in the Manual of Serologic Tests for Syphilis,[18] published periodically by the United States Public Health Service. Treponemal tests are in use in many state public health laboratories and to some extent in local laboratories with the proper facilities. The findings of the SERA Study[19] in which 38 nontreponemal and treponemal tests were evaluated are summarized.

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INTRACARDIAC SURGERY AS VIEWED BY THE MEDICAL TECHNOLOGIST*

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Recent developments in cardiac surgery have made the medical technologist indispensable to the physician in the intelligent management of the patient undergoing cardiopulmonary by-pass. She uses a knowledge of chemistry, hematology, and blood banking to help evaluate the patient's condition during by-pass and in the postoperative period. The purpose of this paper is to discuss the physiology of artificial blood oxygenation and to emhasize the importance of certain laboratory procedures in helping to insure a satisfactory outcome for the patient undergoing open-heart surgery.

What is open-heart surgery or what is meant by cardiac by-pass? Just as the word implies—the heart is opened to allow repair under direct vision in a bloodless field. "Cardiac by-pass" means that the blood is detoured around the heart to allow cardiotomy without interruption of circulation to the patient. A brief review of vascular anatomy (Fig. 1) will reveal that blood enters the heart via two great veins, the inferior and superior venae cavae. These veins empty into the right atrium from which blood is ejected through the tricuspid valve into the right ventricle. This chamber serves as a pump for blood flowing through the pulmonary circuit. From the pulmonary vascular bed, arterialized blood enters the left atrium, goes through the mitral valve into the left ventricle and from there is ejected into the systemic arterial tree. In order to maintain this

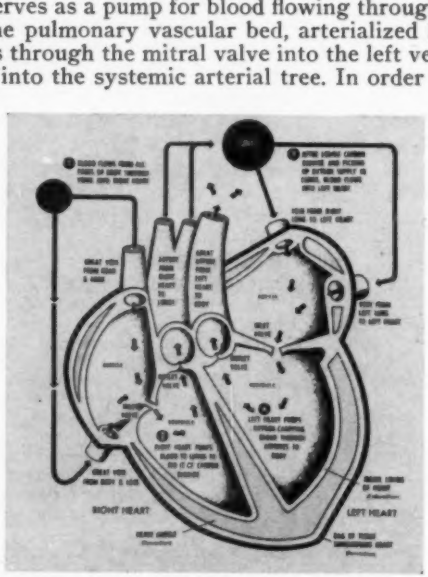

Fig. 1

* Read before the Annual Fall Seminar of the North Carolina Society of Medical Technologists, Raleigh, North Carolina, October, 1959. Received for Publication December 1959.

circulation and still by-pass the heart, blood must be shunted through an extra-corporeal circuit, oxygenated, and pumped back into the arterial system. This is accomplished by the insertion of catheters into the superior and inferior venae cavae and into a systemic artery. Blood drains from the cavae into a chamber called an oxygenator from which it is pumped into the arterial system of the patient. The femoral or subclavian artery is usually selected as the port of entry for the arterialized blood from the oxygenator. When the femoral artery is used, the direction of flow in the aorta is reversed from that occurring under normal conditions.

At the present time, oxygenation of the blood may be carried out in any one of four currently used methods; these are essentially the (1) Bubbler, (2) Screen, (3) Membrane, and (4) Rotating disc. These lungs are descriptively named and the gas exchange is accomplished in the manner imagined from interpreting the name of each particular type. In the usual bubble oxygenator (Fig. 2) blood is mixed with oxygen in a vertical column. The many bubbles which form create a tremendous surface area allowing adequate gas exchange at the blood gas interface. Bubbles are broken by contact with one of a number of anti-foam substances and the blood usually flows through a spiral helix whose length is determined according to the volume of blood flow per minute previously calculated for the perfusion. From this reservoir blood passes through a filter and back to the patient's arterial system.

The screen oxygenator (Fig. 3) works on a slightly different principle. The blood is pumped to the top of a chamber and allowed to flow down a set of finely meshed screens. To insure complete filming or filling of

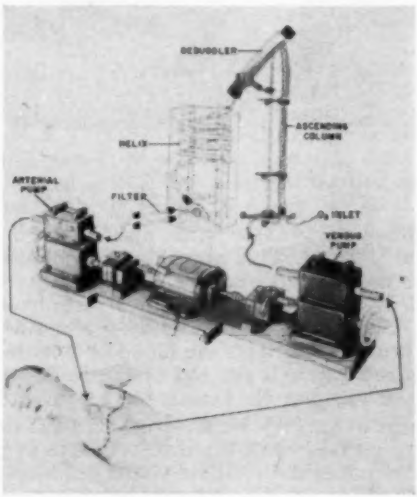

Fig. 2

each hole in the screens, a teflon filmer is pulled up and down in order to wet the screens with the blood. Oxygen is piped in through two openings on the side of the chamber and the arterialized blood leaves the oxygenator through filters to the patient.

The membrane principle allows complete separation of blood and gases by means of a semipermeable membrane. Diffusion of gases occurs through the membrane without allowing any blood-air or blood-gas interface. The most commonly used membranes are made of polyethylene and teflon.

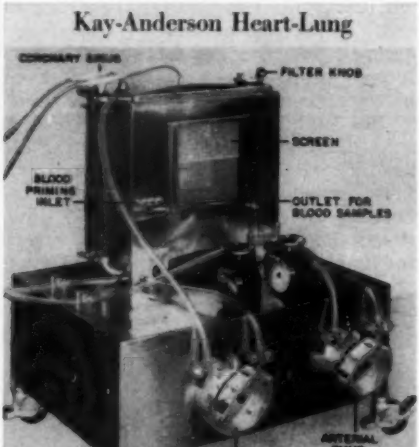

Fig. 3

The fourth and last oxygenator is the disc, or more descriptively, the rotating disc type (Fig. 4). This apparatus is cylindrical in shape and is composed of a metal shaft running through its center upon which convoluted metal discs are mounted with a metallic spacer or washer separating them. This shaft is connected to a motor and rotates the plates which in turn pass through the blood and cause it to film on the disc. Oxygen is piped through the top of the chamber and arterializes the blood filmed on the disc for return to the patient.

In our department of surgery we have used all but the membrane and presently are more pleased with the disc principle in practical application. The bubbler requires the smallest volume of blood for priming and is valued for this advantage. The screen does not require an anti-foam agent as does the bubbler and has proved to be a satisfactory oxygen-

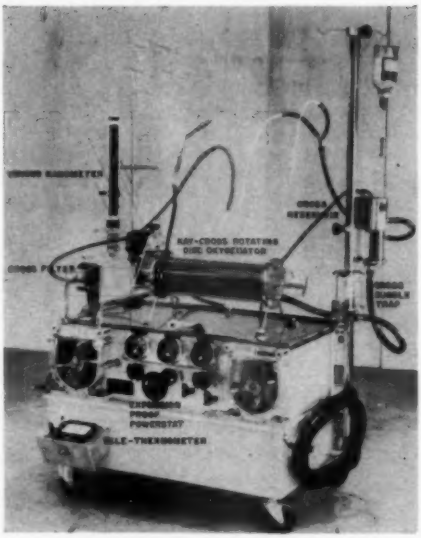

Fig. 4

ator, but was soon replaced by the rotating disc which can more readily be adapted to change in patient size by adding or subtracting discs to meet the blood flow requirements of an individual perfusion.

What hematological changes take place when these various oxygenators are substituted for the lungs and mechanically operated pumps replace the function of the heart? Our main concern, of course, is adequate oxygenation in order to prevent hypoxia with damage to the central nervous system. Oxygen determinations, therefore, are obtained regularly and with the knowledge of the total hemoglobin, per cent saturation is calculated easily. The classical method for ascertaining O_2 content is by Van Slyke gasometric technique. This method involving the release and absorption of CO_2 and O_2 with a saponin-ferricyanide acid solution, sodium hydroxide and hyposulfite solution is time consuming and has been replaced in most laboratories by the spectrophotometric method of Hickman and Frayser using saponin as a hemolyzing agent and reading on a Beckman DU or B against a saturated sample treated in a similar manner.

Plasma hemoglobin levels have been of great concern during the initial use of the different pumps with levels reaching 400 mg. per cent on the bubble oxygenator. These values quickly decrease over a 2 to 24 hour period as the hemoglobin is excreted in the urine. The rotating disc affords another advantage in that it produces little or no destruction to the red cell, thereby keeping the plasma hemoglobin levels within normal or a slightly elevated range.

Coagulation studies are determined in order to ascertain any abnormal bleeding tendencies. The patient is heparinized prior to being connected to the pump in order to render blood incoagulable during the by-pass period. Immediately after disconnection from the pump a coagulant, protamine sulfate or hexadimethrine is given to restore normal coagulability. It is the technologist's role to calculate proper coagulant dosage. This is done with the aid of a titration test in which a sample from the pump which has been primed with heparinized blood is pipetted into a serially diluted set of tubes containing the coagulant. Dosage is calculated after observing the first tube in which clotting occurs. An overdose of protamine can effect continuous bleeding and counteract the intended coagulation.

The occasional development of metabolic acidosis makes pH the most often requested determination during the postoperative period. It is the quickest determination to run and can indicate in a matter of minutes the acid-base status of the patient.

The CO_2 content also is a dependable guide in treating acidosis or alkalosis and is run every two or three hours until stabilization occurs.

Sodium, potassium, and chloride values are helpful in maintaining electrolyte balance but are of less concern than the pH and CO_2 content.

Platelet counts drop severely during by-pass and remain low for several days until they reach normal limits again generally after the fourth to sixth day. At first reduced platelets were thought to be one of the factors causing any bleeding problem that might arise but this hypothesis has been discredited in the case of open heart surgery.

BUN, total protein with A/G ratio, glucose and bilirubin are followed after surgery. Renal shut-down can occur and thereby prolong convalescence.

The hematocrit and hemoglobin are repeated every two to three hours and blood is given when indicated.

This is briefly intra-cardiac surgery as viewed by the medical technologist. It is a role often underestimated by the technologist who does not understand the problems of the surgeon. It is, however, a rewarding field for the person who is cognizant of the importance of her work in the successful care of a patient undergoing intra-cardiac surgery.

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RADIOISOTOPE INSTRUMENTATION: STANDARDIZATION AND CALIBRATION AS APPLIED TO SCINTILLATION SPECTROMETRY*

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In reviewing the texts,[1,2,3,4] manuals, and literature currently in use in the radioisotope laboratory, it is difficult to extract the essential information needed to calibrate and standardize the spectrometers, since this information is either not completely covered or only vaguely so in any one publication. Thus, there is a need for these simple procedures to be compiled in one readily available source. True, there are many chapters devoted to instrumentation, particularly with regard to physics of the electronic instruments; however, it would be most helpful to laboratory personnel if such basic steps were concisely outlined for rapid reference. The purpose of this paper is to take the reader stepwise through the procedures of standardization and calibration, to introduce him to some of the probable errors, and to acquaint him with some of the problems that may develop while using these instruments. Basic physics will not be included in this discussion except where it applies to a particular step of calibration and standardization. It should also be noted that only gamma detectors and gamma emitting radioisotopes are discussed inasmuch as these procedures are used in diagnostic studies.

We are concerned here with two basic pieces of equipment; the scaler and the spectrometer sometimes called the pulse height analyzer. The function of the scaler is to serve as a register and record each pulse as it is received into the machine. The ability of the scaler to record pulses or counts is dependent upon the operational voltage or the Geiger-Mueller Plateau. The theories of the Geiger-Mueller Plateau and its relationship to the operating voltage is well covered by Quimby[4] and associates and a discussion therefore is not considered necessary to the subject at hand.

Operational Voltage

The operating voltage of a scaler and scintillation detector (NaI crystal) is determined in the following manner:

1. A performance source, Cesium,^{137} is placed in front of the detector or in the well.
2. With the voltage switch off and the volt meter control turned to its lowest value, turn the switch on and allow the scaler and detector to warm up for 10-15 minutes.
3. After the warm up period, count the source for one minute at the lowest voltage on the dial and then proceed by increasing the voltage by 50 volt increments, and recording the data.
4. Continue counting for one minute at 50 volt increments. In the beginning there are a few counts, then a rapid increase, followed by a steady rate of counting, this is then followed by a very rapid rate of counts. It is at this point that counting is stopped.
5. Using linear graph paper, plot the counts per minute against the volts and connect the points.

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6. Choose an operating voltage at that point which is one-third of the way up the plateau. Usually the region of the plateau is 200 volts long and as the scintillation detector ages, this region becomes shorter.

7. When using the detector with a scaler, it is necessary to determine the operating voltage for each NaI (Thallium activated) crystal used in detecting gamma emitting radioisotopes. This voltage should be checked at frequent intervals.

Spectrometer (Pulse Height Analyzer)

The function of a gamma spectrometer is to count only those pulses which have a certain energy or "fall" into a well defined energy field. It is this instrument that enables us to count a single gamma-emitting radioisotope. The spectrometer must be calibrated for each isotope that may be used. A day by day standardization of the high voltage is necessary.

A spectrometer is used with a scaler and a timer. The spectrometer and scaler can be combined in one unit and it is primarily this unit which will be discussed. On the combined unit there are certain control knobs that are necessary to its standardization and calibration. These controls are: gain, window (channel width), base level (pulse height selector) and high voltage adjustment.

Gain This control may be found on the front of the spectrometer or on the rear panel with several connectors, fuses, and the power cord. The gain is one of the most difficult controls to explain, since it controls the amplification of the electronic field. Owen[3] describes the gain as the adjustment that regulates the degree of amplification of the pulses arriving at the scaler. We refer to the gain as a degree of sensitivity of the spectrometer when explaining its function to students. The instruction manual that comes with the spectrometer will suggest the gain setting; however, we have used a gain 8 to standardize the high voltage adjustment using a performance standard of Cesium.[137]

Window (channel width) This control sets the upper discriminator in relation to that of the lower discriminator between 0-10 volts so that only certain pulses will be counted. The window and the base level settings are dependent on each other in obtaining the maximum number of counts.

Base level (pulse height selector) This setting is determined by the energy of the radioisotope to be counted and is calibrated from 0-100 volts or 0.000-0.999 m.e.v.

High voltage adjustment This controls the voltage applied to the photomultiplier tube which is housed behind the NaI crystal. Each photomultiplier tube has a given voltage which is determined each day. We check the high voltage adjustment each morning with Cesium[137] and keep a record of the setting. The comparison of the daily settings is an important check on the function of the tube. For example: the high voltage determined with Cesium[137] at .612 m.e.v., gain 8, 10V window, and in a well scintillation counter has been 299 ± 2 volts for the past 6 months. A shift in the high voltage would denote mechanical problems.

Standardization

The first step in setting up the spectrometer is that of standardizing the high voltage, while the second step is to calibrate for the particular

isotope. We use Cesium^{137} as a performance standard due to the long-half life of 37 years. The high voltage adjustment is determined in the following manner:

1. Cesium^{137} is placed in front of the detector or in the well.
2. The following controls are set: base level—.612 m.e.v.; window—10V; and gain—8.
3. High voltage adjustment control turned counter clockwise to zero.
4. Scale selector control reduced (From 512 to 64 or 1000 to 100).
5. With the timer set, turn the high voltage control clockwise from zero until pulses begin to register, with increase of voltage the pulses will increase to a rapid rate and then decrease giving a characteristic peak.
6. When the range of the maximum number of counts is located, turn the scale selector to its usual position and count for one minute at one volt increments until the peak is located. This usually is over a range of 10 volts.
7. The high voltage setting is that voltage where the greatest number of pulses register using Cesium^{137} as a performance source. It is the voltage which must be applied to the photomultiplier tube to obtain the maximum number of counts from the source.

To facilitate the day to day standardization it is helpful to reduce the scale selector so that less pulses are necessary to make the mechanical scaler register; therefore, producing more "noise" by clicking. For example: for a binery system, the scale selector would be reduced from 512 to 64 so that for every 64 pulses received a number would click on the register. In this manner the range of the high voltage can be picked out by the intensity of the clicking, then proceed by doing one minute counts. Some instruments are equipped with hummers to facilitate such standardization.

Calibration

Once the high voltage has been determined, the next step is to calibrate the spectrometer with the specific radioisotope by doing gamma-ray spectra energy curves. The specific gamma radiation energy in m.e.v. of each radioisotope may be found on the chart of the "Nuclides and Isotopes."* Following is a list of the radioisotopes more commonly used in the clinical laboratory:

Isotope	Gamma Radiation Energy in m.e.v.
Cr^{51}	.32
I^{131}	.36
Cs^{137}	.662
Co^{58}	.81; 1.64
Co^{60}	1.33; 1.17
Fe^{59}	1.10; 1.29

Using a differential spectrometer with a window (lower and upper discriminator) the maximum number of counts must be received at a base level corresponding to the gamma radiation energy in m.e.v. of the particular isotope.

For example: the high voltage has been determined using a gain 8 with Cesium^{137} as performance standard. Therefore, gain 8 is used for

* "Nuclides and Isotopes," 4th edition, April, 1956, General Electric Company, Schnectady 5, New York.

the calibration of any radioisotope having a gamma radiation energy in the range of 0.0 m.e.v. to 1.0 m.e.v. On the basis of this fact the gain may be reduced from 8 to 4; thereby, expanding the electronic field to range of 0.0 m.e.v. to 2.0 m.e.v. On the other hand, if the gain was increased from 8 to 16 there would be a corresponding change in the electronic field to a range of 0.0 m.e.v. to 0.5 m.e.v.

If however the high voltage has been determined by using a gain 4 with Cesium^{137} then a gain 4 is used for any isotope having a gamma radiation energy of 0.0 m.e.v. to 1.0 m.e.v. Thus, it would be necessary to count specimens containing isotopes with gamma radiation energy greater than 1.0 m.e.v. on a gain 2. Owens[3] states that if the setting is at the extreme of the dial, there is a minimal amplification while at the other extreme of the dial there is a large increase in pulse heights.

When using Cesium^{137} as the performance source, the student must bear in mind that whatever gain setting is used for the determination of the high voltage that gain setting is assigned the "energy value" of 0.0 m.e.v. to 1.0 m.e.v.

The window or upper discriminator is set in accordance with the m.e.v. of the radioisotope and the base level or lower discriminator. These settings are interdependent and if improperly used will and can reduce practically all counts. It is most important to have the peak of the pulse height in the middle of the window and if we arbitrarily assign m.e.v. values to the window settings, this will facilitate understanding the determination of the spectra-energy curves. Bear in mind that this is an arbitrary assignment and that the theory governing the discriminators may be found in the operation manual that comes with the spectrometer. In this manner the window settings of 0-10 volts may be assigned the energy value of 0-.100 m.e.v. For example: Cesium^{137} has a pulse height gamma radiation energy of .662 m.e.v., when setting the base level at .612 m.e.v. with a 10 V (.100 m.e.v.) window and a gain 8, the pulse height peak should be in the middle of the window so that half of the window setting added to the base level setting equals the gamma radiation energy in m.e.v. In this example, one half of the window setting equals .050 plus the base level of .612 equaling the energy of .662 m.e.v. for Cesium^{137}. These calculations are very important in plotting the spectra-energy curves.

Using a gamma differential spectrometer one can determine the spectrum-energy curve for Cesium^{137} as follows:

1. High voltage adjustment set (determined as previously described).
2. Base level turned counter clockwise to zero.
3. Window 10 V.
4. Gain 8 (range 0.0-1.0 m.e.v.)
5. Place the Cesium^{137} in front of the detector or in the well.
6. Set the base level at zero and count for one minute, then continue by turning the base level control by 50 m.e.v. increments and counting for one minute until the base level setting is at .550 m.e.v., then reduce to 10 m.e.v. increments to a base level of .600 m.e.v. then proceed at 1 m.e.v. increments until the peak of activity is reached and then decreases.

If everything is functioning properly the greatest number of counts will register at a base level of .612 m.e.v.

7. On linear graph paper plot the counts per minute against the energy in m.e.v., accounting for the window by adding .050 to the energy.

8. Draw the curve and the peak should be at .662 m.e.v. See Fig. 1.

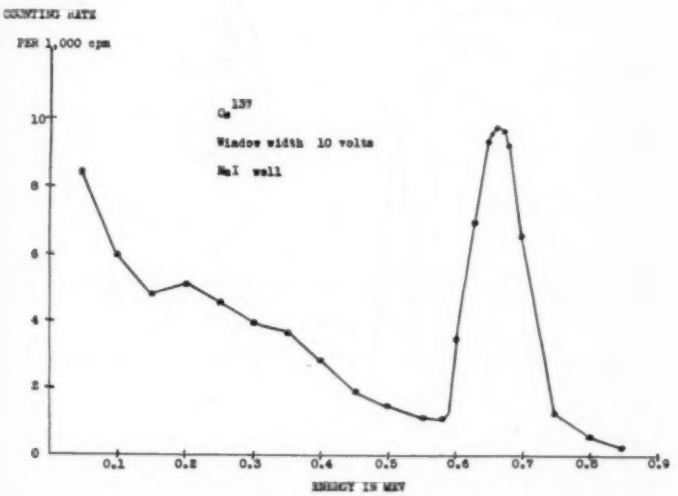

Fig. 1. Gamma spectrum energy curve of Cesium[137], characteristic radiation 0.662 m.e.v.

The window width can be varied in order to preclude the counting of certain pulses. However, the lower and upper discriminators should be adjusted together in such a manner in order that the peak of the pulse height may be in the center of the window. Quimby[4] and associates point out that the wider the window the poorer the resolution of the gamma-ray peaks, but the higher the sensitivity. The narrower the window, the greater the resolution but the smaller the sensitivity. In Fig. 2 the same specimen of Chromium[51] was counted at 10 V, 4 V, and a 1 V window settings with the proper adjustment of the base level setting to show a comparison of the sensitivity and resolution. Nevertheless, in the clinical laboratory when patients are given radioactive tracer doses, it is advisable to keep the tracer dose low and work with high sensitivity and low resolution, thus, a 10 V window is more commonly used in counting specimens.

The spectra-energy curves for Iodine[131] and Chromium[51] are plotted in a similar manner as Cesium[137] but taking into account the different energy of the isotopes. Figs. 3 and 4.

In determining the spectra-energy curves of Iron[56] and Cobalt[60] it is necessary to account for two energy peaks. These isotopes have two pulse height peaks in a relatively narrow range of energy.

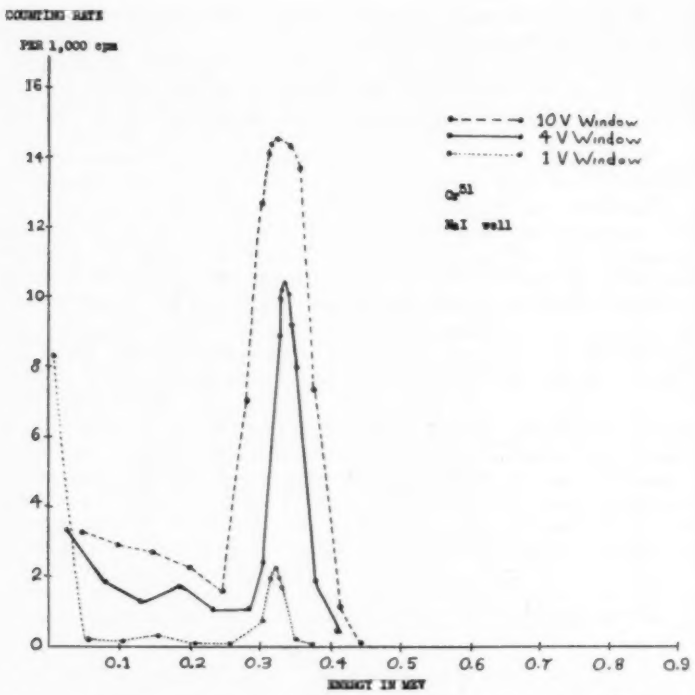

Fig. 2. Comparison of the sensitivity and resolution of a sample of Chromium^{51}. The 1 volt window width setting gives the best resolution and the least sensitivity; the 10 volts window width setting gives the best sensitivity.

Using a gamma differential spectrometer one can determine the spectra-energy curves of Iron^{59} and Cobalt^{60} as follows:

1. High voltage adjustment set (determined as previously described).
2. Base level turned counter clockwise to zero.
3. Window 2 V.
4. Gain 4 (range of 0.0-2.0 m.e.v.)
5. Place the Iron^{59} or Cobalt^{60} in front of the detector or in the well.
6. Set the base level at zero and count for one minute, then continue by turning the base level control at 50 m.e.v. increments and counting for one minute until the base level is at .500 m.e.v., then reduce to 5 m.e.v. increments to a base level of .545 m.e.v., continue at 1 m.e.v. increments to a base level of .555 m.e.v., increase by 10 m.e.v. increments to .645 m.e.v. At the second peak of activity use 1 m.e.v. increments past the range of activity and then continue using 10 m.e.v. increments until the curve levels. See Figs. 5 and 6.

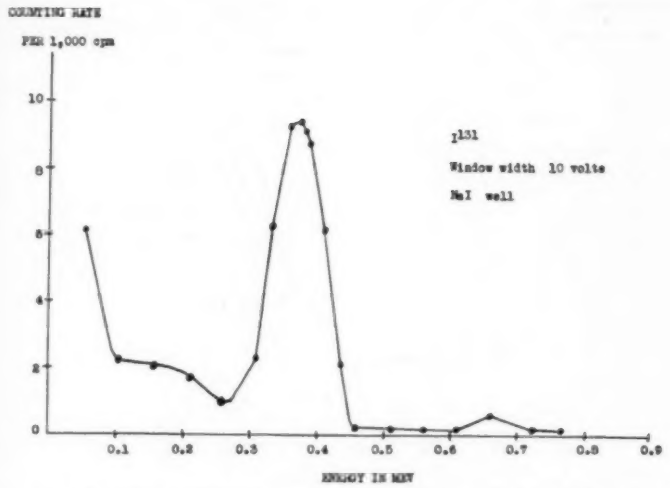

Fig. 3. Gamma spectrum energy curve of Iodine^{131}, characteristic radiation 0.360 m.e.v.

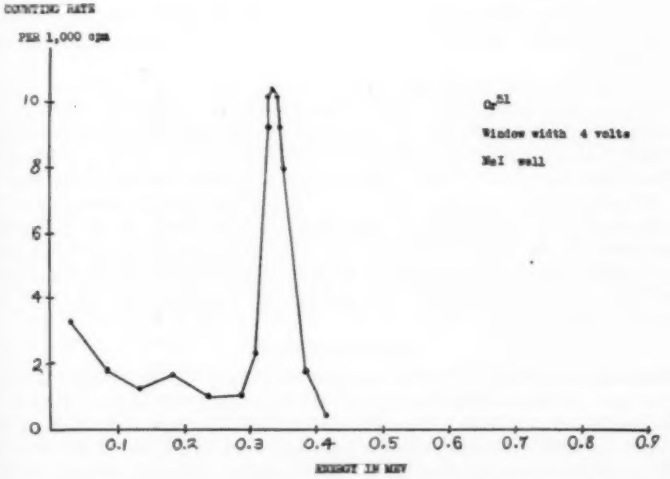

Fig. 4. Gamma spectrum energy curve of Chromium^{51}, characteristic radiation 0.320 m.e.v.

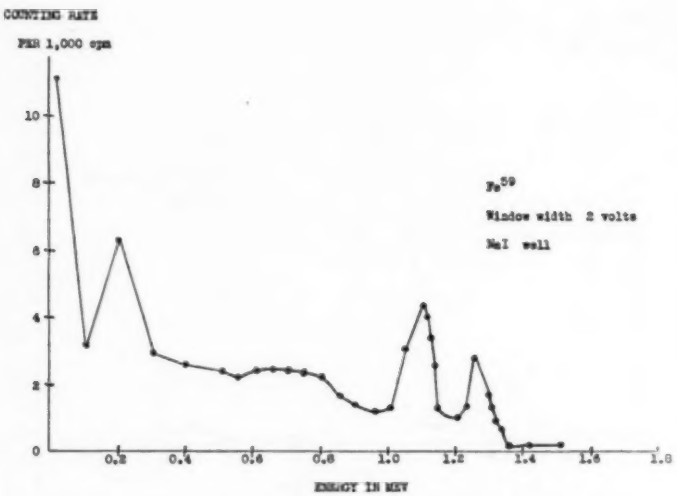

Fig. 5. Gamma spectrum energy curve of $Iron^{59}$, characteristic radiations 1.10 and 1.29 m.e.v.

7. On linear graph paper plot the counts per minute against energy in m.e.v. and accounting for the window and gain settings. In order to account for the gain; take the total of the result of adding the base level and one half of the window and multiply by 2. For example: if the first peak of activity is .550 m.e.v. add half of the window setting of .010 to the base level and multiplying by 2 equals 1.12 m.e.v. for the pulse height peak.

8. Draw the curve on linear graph paper and the $Iron^{59}$ or $Cobalt^{60}$ should have two peaks; one at 1.1 m.e.v. and the other at 1.3 m.e.v.

It should be noted that a 2 V window was used in determining the $Iron^{59}$ and $Cobalt^{60}$ spectra-energy curves which was different from that described for $Cesium^{137}$. This was done in order to describe the two peaks of gamma-ray energy which are given off by these isotopes. If a 10 V window had been used, the peaks would have been difficult to differentiate. In the practical aspect of counting specimens, it is necessary to utilize the two energies of $Iron^{59}$ and $Cobalt^{60}$ so that the upper discriminator is removed by the means of turning the control from window to base.

Standards

Calibrated radioactivity reference sources play as important a role in radioisotope determinations as do standard solutions in chemical analysis. Calibration standards are distributed by the commercial firms several times a year but due to the short half-life, are limited in their use. For this reason one of the long lived radioactive materials such as $Cesium^{137}$ is used as a performance standard in checking equipment.

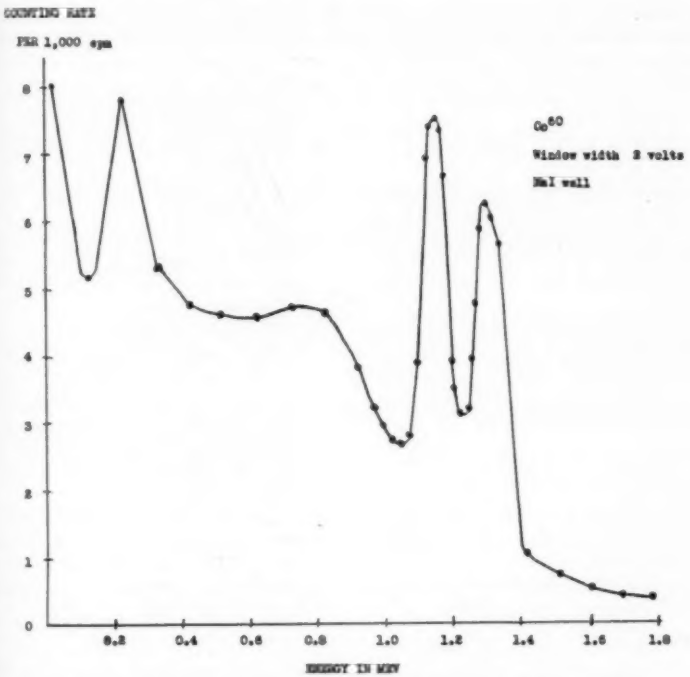

Fig. 6. Gamma spectrum energy curve of Cobalt^{60}, characteristic radiations 1.17 and 1.33 m.e.v.

Quimby[4] and associates point out, however, that a performance standard is not the same as a calibrated standard of the particular isotope. They further recommend that every standardization set-up should be recalibrated with standard samples as frequently as such standards can be obtained, at least twice a year.

A simulated or "mock-iodine" source is available for use as a performance and calibration standard. This standard is a combination of Barium^{133} and Cesium^{137} yielding a gamma-ray spectrum similar to that of Iodine^{131} and having a half-life about 10 years. The standard can be purchased from the distributors of radioisotope equipment.

Sources of Error

In using the electronic instruments of the radioisotope laboratory, as with using other laboratory instruments—there must be a constant alertness for mechanical difficulties. As a rule these are easy to detect in the electronic equipment in that the lights will not turn on or pulses will not register. On the other hand, there may be mechanical problems that produce mechanical errors in your results which may go undetected.

For example, there may be a shift in the window width or one of the discriminators may be defective. Some of the most common mechanical defects and probable corrections are:

1. Interpolation light out in binery system.	1. Check glow lamps and if necessary change, check tubes
2. Master switch light out	2. Check light, then connections and fuses
3. Instrument will not count pulses but will count on 60 cycle test	3. Check connections and H. V. Adjustment
4. Using standard sources: usual number of counts recorded for one source but not for the other	4. Check base level and window width settings; if correct, then may have defective tubes in the window mechanism
5. Instrument counts at an irregular rate for short periods of time while counting specimens —difficult to duplicate within 2 standard deviations	5. Spurious counts due to line noise—may be from other electrical equipment. May be tube becoming defective. Rule out if the counts are coming from other electrical equipment—if the irregular counts continue call for service
6. Instrument counts at random whether or not the detector is connected	6. Spurious counts from within the instrument due to "noise" or in the connections
7. Instrument counts with one detector and not another	7. Defective pre-amp or photomultiplier tube

We have found it advisable to keep some of the tubes on hand for replacement. Before calling for service be sure and check all connections and settings of the controls, for often the problem may be technical rather than mechanical. The connections from the detector to the instrument are screw type rather than plug in; therefore, special care must be used in setting up the instrument when changing from one detector to another. When service is necessary one can often outline the problem on the telephone, and minor adjustments can be made without losing too much time, or the use of the equipment.

Discussion

Just as one does not or most certainly should not use a spectrophotometer without prior standardization and calibration, so one should not use the gamma spectrometer without proper standardization and calibration. In the utilization of radioisotopes we know the energy just as we know the proper wave length of certain color controls in utilizing the spectrophotometer.

In the calibration procedures, it is assumed that the results of the calibration will correlate with the known spectrum-energy curve of the particular isotope. If the spectrum-energy curve does not correlate, the high voltage is redetermined and the settings of the base level, gain, and window are rechecked. If the spectrum-energy curve, after these determinations still does not correlate, then there is a mechanical problem.

The interrelationship of the base level, window, and gain enables us to choose the best resolution and sensitivity when counting specimens. This relationship is also important when it is necessary that background be reduced. Such reduction can be achieved by reducing the window and setting the base level accordingly.

The gamma-ray differential scintillation spectrometer has been discussed without any mention of the equipment using integral discriminators. The integral spectrum differs in appearance from the differential spectrum in that the former presents a smooth sloping curve while the latter shows a peaked curve. For additional information pertaining to integral discriminators you should refer to the "Handbook for Scintillation Spectrometry."[2]

Summary

1. The procedures used in the standardization and calibration of a gamma-ray differential scintillation spectrometer are described, and the spectra-energy curves of the more common diagnostic radioisotopes are presented.

2. A brief discussion of the standards recommended for use in the radioisotope laboratory is presented.

3. Some of the probable mechanical errors are listed. Emphasis is placed on checking and rechecking all settings and connections before calling for service.

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A SIMPLE METHOD OF PREPARING BOVINE SERUM AS A CONTROL FOR DETERMINATION OF BLOOD GLUCOSE WITH ANTHRONE*

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Introduction

In the past few years the value of using control serum to improve the accuracy of tests in a clinical chemistry laboratory has been demonstrated amply (Henry, 1959). Although several brands of commercial control serum are available, they are relatively expensive for daily laboratory use. In addition, some have values which have been determined in other laboratories, often by different techniques.

This paper describes a simple, inexpensive method for the preparation of bovine serum which can serve a double duty: as control, and as a standard in quality control of blood or serum glucose determinations. In this instance the method has been applied to the glucose determination with anthrone.

Material and Methods

Preparation of control serum:

1. Serum from fresh steer blood (obtained from the slaughter house and transported in a sterile gallon jar) is separated, by allowing the blood to clot during overnight refrigeration. Each gallon of blood yields approximately 1000 ml. of serum.
2. Glucose is eliminated from the serum with baker's yeast:
   a. Obtain fresh, active Fleischmann's yeast cakes; use approximately one cake for each 300 ml. of serum.
   b. Wash yeast in distilled water with successive centrifugations. Use a 90 ml. centrifuge tube for each yeast cake. Three to four washings are sufficient to obtain a clean product.
   c. Using sterile glassware, transfer serum to a flask by suction.
   d. Mix clean yeast with steer serum and incubate for one hour at 37° C. Agitate serum by shaking occasionally during incubation period.
   e. Separate serum from yeast by centrifuging at 2000 RPM for 20 minutes.
   f. Add sufficient sodium fluoride to make a 1 per cent solution to inactivate any glycolytic enzymes present.
   g. To ascertain that glucose has been completely destroyed by yeast, make trial glucose determinations on the serum.
3. **Preparation of glucose standard:**
   a. Concentrated serum glucose standard, 10 percent: Place 10 gm. dried glucose in a 100 ml. volumetric flask, and add sufficient glucose-free serum to make the required amount.
   b. Working glucose serum standard, 100 mg per cent: Dilute 1.0 ml. of concentrated glucose standard to 100 ml. with glucose-free serum. (For storage transfer approximately 2 ml. working

* Received for publication October, 1960.

standard to each of a series of 5 ml. test tubes, seal with Parafilm, and store in freezer between —20° and —50° C.)

c. Thaw serum before using; mix well. (If kept between 2 and 6 degrees C, serum may be re-used within 48 hours in the non-frozen state. Serum standard may be used safely after being stored frozen for at least ten months.)

4. **Preparation of curve and quality control chart:**

To prepare the standard curve the following dilutions are recommended, using the original stock solution containing 10 per cent glucose and diluting with glucose-free serum.

1. 1.0 ml. stock solution diluted to 25 ml. = 400 mg%
2. 0.2 ml. stock solution diluted to 10 ml. = 200 mg%
3. 0.1 ml. stock solution diluted to 10 ml. = 100 mg%
4. 0.05 ml. stock solution diluted to 10 ml. = 50 mg%
5. 0.02 ml. stock solution diluted to 10 ml. = 20 mg%

The control chart was prepared in the usual manner, as described by G. R. Kingsley (1956).

Anthrone Method of Glucose Determination

Reagents:

1. Protein Precipitants:
   A. Acid zinc solution: Place 4.0 gm. of $ZnSO_4$ and 15 ml. of 1.0 N H_2SO_4 in a liter volumetric flask and make up to volume with distilled water.
   B. Sodium hydroxide, approximately N/6: Prepare from saturated stock solution (Dilute 9.8 ml. to 1 liter). Adjust the concentration so that exactly 6.41 ml. is required to titrate 25 ml. of acid-zinc solution, using phenolphthalein as indicator. Stir continuously throughout the titration to avoid a false end-point.
2. 32N H_2SO_4: Place 120 ml. of distilled water in a 2 liter Erlenmeyer flask. Add slowly one liter of reagent grade sulfuric acid, stirring constantly. Cool to room temperature before using.
3. Anthrone reagent: Place 500 mg. of anthrone and 2.5 gm. of Thiourea in a 250 ml. volumetric flask and make up to volume with 32N H_2SO_4. Refrigerate in dark bottle. Prepare every two weeks.

Equipment:

1. Seligson pipette (0.2 ml. calibrated tip and 25 ml. burette). (Seligson, 1957)
2. Chaney-type 5 ml. syringe pipette, calibrated to deliver 4.0 ml.

Procedure for Blood Glucose Determination:

1. Prepare a 1:50 filtrate of the unknown blood or serum and of the 100 mg% serum glucose standard as follows:
   A. Transfer 200 lambda of the blood or serum and 7.8 ml. of acid-zinc solution into a test tube using the Seligson pipette. (The difference between initial and final burette readings is 8.0 ml.)
   B. Filter through a plug of glass wool. (Do not use filter paper.)
2. Pipette 4.0 ml. of anthrone reagent (syringe pipette) into each of three 18 x 150 mm. test tubes. Label the tubes: Blank — Standard — Unknown. Over the anthrone reagent layer 2.0 ml. of water, control serum filtrate, and unknown filtrate respectively.

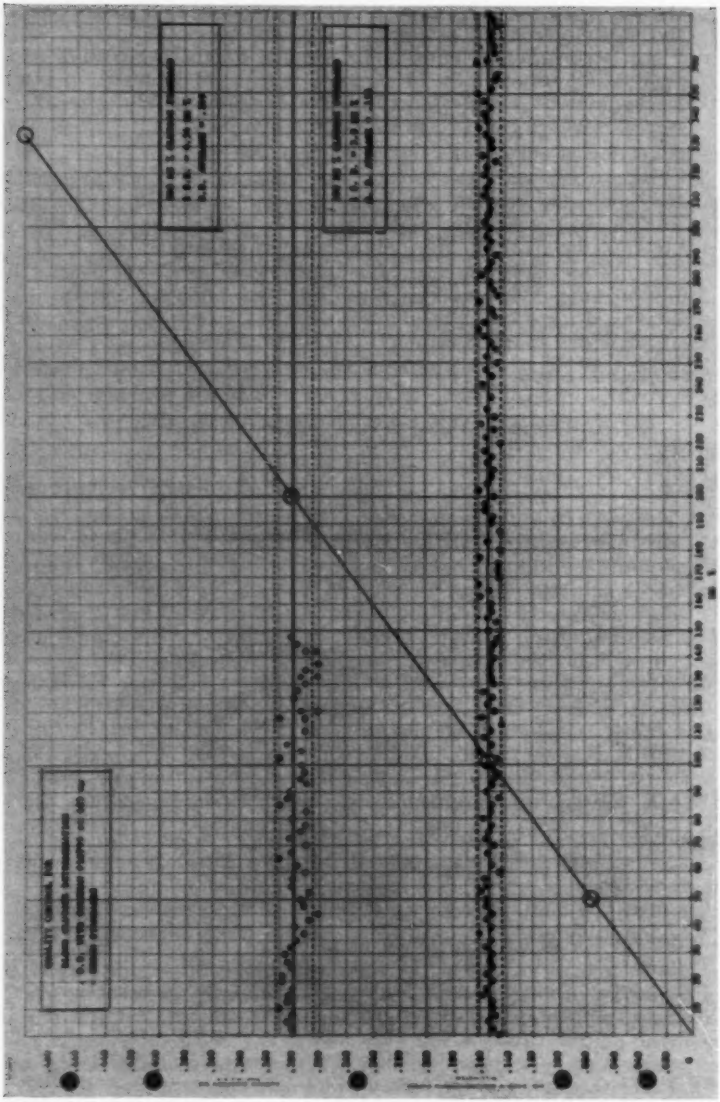

Figure 1—Quality Control chart and standard curve.

3. Mix the content of each tube thoroughly by tapping, and place the tubes in boiling water for 12 minutes.
4. Cool for at least 2 minutes in cold running tap water.
5. Read the O. D. of Standard and Unknown within 15 minutes at 625 m μ., setting the instrument at zero O. D. with the Blank.
6. Determine the mg% glucose in standard and unknown from the standard curve. Plot the result in O.D. for the standard on the control chart. If this value falls outside the limits set (plus or minus 2 S.D.), the test must be repeated. If the value for any unknown exceeds 400 mg%, the test is repeated using 1 ml. filtrate and 1 ml. of water and the result multiplied by two.

RESULTS

Fig. 1 shows the quality control chart obtained in the Clinical Laboratory of Children's Hospital, San Francisco, comprising the determinations of 6 technicians, over a period of ten months. The standard deviation was .009 O.D. (3.0 mg% glucose).
(The 200 mg% serum standard shown on the chart was used for some time along with the 100 mg% standard. Since the 100 mg% value is nearer to the normal range, and since the standard curve was shown to follow Beer's Law up to 400 mg%, the use of the 200 mg% control standard was discontinued.)

Fig. 2. The effect of acid concentration on the rate of development and stability of color is indicated in Fig. 2. The boiling time was selected from the center of the plateau of each curve to minimize the effect of slight variation in boiling time with large numbers of determinations. At present 32N H_2SO_4 (curve A) is being used, since the midpoint of the plateau occurs at 12 minutes, which is a more convenient time.

Figure 1—Quality Control chart and standard curve.

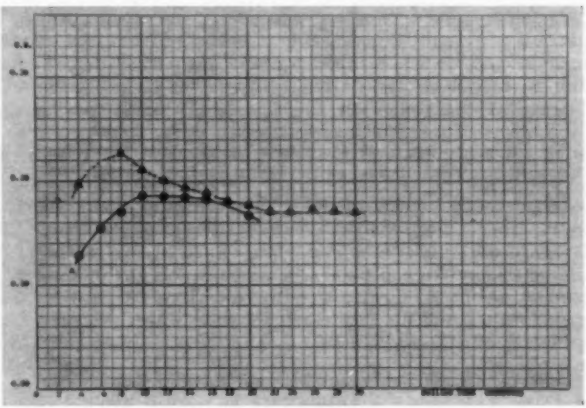

Figure 2—A. Anthrone Reagent 32N H_2SO_4
B. Anthrone Reagent 34N H_2SO_4

The stability of the frozen control serum was checked until the supply was exhausted (10 months). No significant change in glucose content was found. No change was found in the glucose content of thawed control serum after 5 days refrigeration or at room temperature.

Discussion

The use of a single serum to serve both as standard and control has obvious advantages:

1. It provides an exact control subjected to the same technique and reactions as the unknown. Inaccuracies which might occur during precipitation or filtration, as well as deterioration and contamination of protein precipitating reagents are easily detected.
2. The prepared product is much less expensive than the presently available commercial control serums.
3. Preparation obviates the use of commercial control serum whose values have been determined elsewhere, often with the use of different equipment and techniques.
4. The combination of standard and control serum lends itself well to the establishment of quality control necessary to obtain the highest possible accuracy.
5. The control serum will remain stable and uniform for long periods; the preparation is reproducible, obviating the necessity of preparing a new control chart for each batch. It can also be prepared in large quantities.
6. The method adapts easily to the addition of "recoveries" and may be used to investigate refinements of different techniques in regard to specificity for glucose.

The following should be noted regarding the anthrone method of glucose determination: stabilization of the anthrone reagent with thiourea (Roe, 1955) is required to produce consistent results from day to day to permit its use in quality control. Since paper fibers hydrolize in strong acid to form glucose and produce artificially elevated results, filtration through glass wool is employed. Several brands of reagent grade sulfuric acid were found to be unsuitable for the preparation of the anthrone reagent due to discoloration of the reagent on storage. This difficulty has not been encountered with the use of Baker's Analyzed Sulfuric Acid.

Because of its high viscosity, accurate pipetting of the anthrone reagent with drainage-type pipettes is difficult and time-consuming. In the authors' laboratory a Chaney-type syringe pipette is used to dispense 4 ml. aliquots of the reagent into a rack full of test tubes at one time. The tubes are then tightly capped with Parafilm and refrigerated until needed. Using the syringe pipette, 40 aliquots of anthrone reagent may be dispensed in about 10 minutes, with less than 0.01 ml. error. On the average, values obtained by this method were 3 mg. per cent lower than those employing the Nelson method, and 16 mg. per cent lower than those using the Folin-Wu method.

Summary

A. A standard and control have been combined in one medium (bovine serum) by means of a simple and inexpensive method. The control

chart indicates the precision obtained with this control serum in the determination of blood glucose.

Several advantages of its daily use in the clinical laboratory have been described.

B. Several factors affecting the precision of blood glucose determination with anthrone have been discussed.

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ABSTRACTS

CALLING THE LABORATORY

VII—THE ERYTHROCYTE SEDIMENTATION TEST

The sedimentation rate is not only the oldest test in clinical pathology, but it is also the most general in its applicability. For practical purposes the test indicates the extent of tissue destruction and exudation. Thus a raised sedimentation rate is not an indicator of any one disease or group of diseases but of a pathological process which may accompany a wide variety of medical, surgical or gynecological conditions. It is a preliminary sorting test, in the light of which other pathological and clinical findings may be assessed. The decrease in sedimentation rate which occurs during healing gives objective evidence of the success of treatment and thus assists in prognosis.

The basic principle of the test is that in certain pathological conditions involving a degree of tissue damage, exudation and reaction, the plasma protein pattern is altered, giving an increased tendency to rouleau formation. The test is quantitative. No precise definition of the rouleau-forming factor can be given. Fibrinogen is important and the globulins also play a part. Albumin has the reverse effect. The resulting rate of fall is thus evidence of a summation of effects and reflects a pattern of plasma protein constitution rather than increase of a single constituent.

If the blood is diluted with one part of isotonic citrate solution to four parts of blood, as in the Westergren method, anemia has little influence and may for practical purposes be neglected.

The Westergren is so widely used internationally that its results have a special value for comparative studies. However, the rouleau-forming effect is probably weakened by dilution. For this reason, in some laboratories a method such as the Wintrobe, using undiluted blood, is performed in addition to the Westergren method to detect minor degrees of abnormality. Gibson, H. J., Area Pathologist, Bath, England. *The Practitioner,* No. 1105, Vol. 185, July, 1960, pp. 107-109.

Continued on Page 100

A SIMPLE METHOD OF DETECTING PHAGE TYPABILITY OF STRAINS OF STAPHYLOCOCCUS AUREUS*

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Phage typing of staphylococcal strains has been an important epidemiological tool in many hospital laboratories. A simple method of detecting phage typable strains could save much time in laboratories where phage typing is carried out on large numbers of cultures. Graber *et al*[3] has presented evidence that the opalescent zone produced around colonies of staphylococci which are grown on egg yolk media could serve as an index to phage typability. Using a larger number of strains than was used in that earlier report, similar work has been carried out in this laboratory.

Materials and Methods

The strains of staphylococci used in this study were isolated from a wide variety of clinical sources. Coagulase production was determined by the slide method. All negative strains by this method were retested by the tube method, using out-dated human plasma obtained from the hospital's blood bank. Controls were run with each test.

Phage typing was carried out using the method of Blair and Carr[1] with the exception that the phanges were applied to the seeded plates with a multiple-typing apparatus, which was designed in this laboratory and has given excellent results. Antibiotic sensitivity determinations were made, using both the single disc method and the tube dilution method.

The egg yolk medium was prepared by the method described by Graber *et al.*[3] A reaction was termed positive when an opalescent zone appeared immediately around the colony after 24 hours incubation at 37° C.

Results

A total of 993 coagulase positive *Staphylococcus aureus* cultures were studied. Table I shows the correlation between the production of an opalescent zone around the colony on egg yolk medium and the ability to be phage typed. The results showed that 850 of the 879 egg yolk positive cultures were phage typable. Only two of 144 strains which were egg yolk negative were lysed by the typing phage used. Of these strains, one was lysed by phage 44A and the other by phages 52/80/81.

TABLE I

Correlation Between Staphylococcal Phage Typability and Opalescence Production on Egg Yolk Medium

No. Cultures	No. Phage Typable	No. EYP*	No. EYN** Strains Which Phage Typed
993.....	850	879	2***

*EYP Egg yolk positive.
**EYN Egg yolk negative.
*** One strain was lysed by phage 44A; the other, by 52/80/81.

† This study was supported in part by a grant from the Research and Development Command, U.S. Army Contract No. DA-49-007-MD-959.
* Received for publication Dec. 1960.

Table II shows the correlation between coagulase production and the production of an opalescent zone on egg yolk media. Evidently, the egg yolk reaction alone cannot be used to detect coagulase positive strains of staphylococci. While 88 per cent of the coagulase positive strains were egg yolk positive, 12 per cent were not. More over, 52 per cent of the coagulase negative strains also were egg yolk positive.

There was no correlation between the antibiotic sensitivity and the egg yolk reaction.

TABLE II

Relationship Between Staphylococcal Coagulase Production and Opalescence Production on Egg Yolk Medium

COAGULASE POSITIVE			COAGULASE NEGATIVE		
No. Cultures	No. EYP*	Per cent	No. Cultures	No. EYP	Per cent
993.....	879	88	27	14	52

* Egg yolk positive.

Discussion

The results found in this laboratory agree with those found by Graber *et al*[3] in that staphylococcal strains which produce opalescent zones around the colonies grown on egg yolk medium are likely to be phage typable; whereas, those that do not produce the zone are very rarely phage typable. There were more egg yolk positive strains than phage typable strains, indicating that perhaps these strains might have been phage typed if a wider range of typing phage had been used.

Only 2 of 114 egg yolk negative strains were phage typable, which indicated that the egg yolk reaction could be a good screening test of phage typability. It is interesting to note that the strains which Graber *et al*[3] reported as being phage typable but egg yolk negative were also lysed by phage 44A. They postulated that the old age of the cultures might have been responsible for this. However, we found that this probably is not the case, since many of the strains which we tested were two to three years old; and we had exact correlation between egg yolk negativity and nonphage typability. The two strains which did not show this correlation in our laboratory were fresh isolates.

Even though 88 per cent of the coagulase positive strains produced a positive egg yolk reaction, this reaction cannot be used as a specific indicator of coagulase production because 52 per cent of the coagulase negative strains also gave a positive egg yolk reaction. We also found no correlation between the egg yolk reaction and the susceptibility to antibiotics, since both resistant and sensitive strains yielded varied reactions on the egg yolk medium. These results agree with Graber *et al*[3] but not with those of Gillespie and Adler.[2] The latter authors reported a correlation between a negative egg yolk reaction and penicillin resistance. They also found no egg yolk positive strains among the coagulase negative strains that they studied.

Summary

A good correlation was found between egg yolk negativity and the inability of staphylococcal strains to be phage typed. There was no correlation found between the egg yolk reaction and coagulase production or antibiotic sensitivity.

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ABSTRACTS

ON DETERMINING THE PACKED CELL VOLUME

The technical simplicity of estimating the packed cell volume seems to arouse a false sense of security in its reproducibility. It is generally held to be less subject to technical artefact than measurement of hemoglobin concentration. Yet when the MCHC of the same patient has been determined 2-3 times per week over a month or so and fluctuations have occurred, they have been due to a variation in the figure recorded for the packed cell volume and not in the figure obtained for the hemoglobin concentration.

Two main sources of error have been found: errors in reading the hematocrit tube and errors resulting from failure to achieve uniform and adequate packing of the red corpuscles.

For reading, the hematocrit tube was kept vertical by using the Wintrobe stand; the reader's eye was kept at the same level as the top of the packed cell layer. Magnification adds precision to the reading, since it is then possible to make an approximate allowance in admixture of red with white corpuscles when separation of the two is not sharp. Under these conditions, the level of the packer cell layer can be read to within a half or quarter of a division.

According to Wintrobe (1956), 30 min. at a force of 2,264 G is required to produce so-called "complete" packing. Instead of this, however, the figure of 3,000 r.p.m. for 30 min. is too often all that is thought important. Even a centrifuge expressly designed for determination of packed cell volume may be fundamentally unsuitable for the purpose.

When packing is incomplete, the MCHC tends to be generally a little lower than the normal 32-36%. Hutchison, H. E., Department of Haematology, University and Western Infirmary, Glasgow, Scotland. *Journal of Clinical Pathology*, 13, 6, 1960, p. 529.

Continued Page 111

MEDICAL TECHNOLOGY TRAINING: A FOLLOW-UP STUDY OF GRADUATES*

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INTRODUCTION

Objectives of the Study

Interest in medical technology education has been intensified by the evidence of need for laboratory workers in greater numbers, as the National Committee for Careers in Medical Technology (8) has pointed out, and with better training, as studies of the accuracy of laboratory analyses indicate (1, 2, 11, 12). Therefore it was thought that an appraisal of the effectiveness of one medical technology training program in preparing students for this profession would be desirable. The device chosen for this purpose was that of a follow-up study of former students, since it has been successfully used by educators in this type of evaluation in fields such as home economics (9), teacher education (6), agriculture (3 and 5), and nursing (4 and 7). Using a questionnaire sent to graduates of the program, the investigation was designed to achieve the following objectives:

1. To find out what kind of professional experience graduates have had.
2. To determine how they appraise the effectiveness of their professional training based upon their practical experiences.
3. To elicit their evaluation of the worth of specific methods of instruction and of program organization.

Description of the Program Being Studied

The medical technology program under consideration was organized as an undergraduate curriculum in the College of Arts and Sciences, leading to a Bachelor of Science degree in four years, with eligibility to take the Registry examination of the ASCP (American Society of Clinical Pathologists). The academic, nonprofessional requirements for the Bachelor of Science degree, for the Department of Pathology under whom the curriculum was offered, and for the Board of Registry of the ASCP were completed in eight quarters. This was followed by one calendar year of instruction in the four main laboratory divisions, with one department per quarter offering lectures by the faculty of the Department of Pathology and laboratory instruction by supervisors from the clinical laboratories. A lecture course in Clinical Pathology with the medical students and a weekly seminar prepared and presented by the students were also given during this clinical year. Laboratory instruction was given in both the clinical laboratories and in separate student laboratories. Notebooks were usually assigned for student preparation and subsequent evaluation, and a given number of determinations on specially prepared samples were required as "check-offs" before tests might be performed on patient specimens. Library research assignments were used very occasionally in the courses, and also in preparation for the student seminar. The class as a whole was given instruction in each new or special procedure by means of demonstrations by the medical

* First Award Educational and Procedural Techniques, Scientific Products Foundation, 1960. Read before the 28th Annual Convention of ASMT, Atlantic City, New Jersey, June 1960.

technologist instructor before students performed them individually in the laboratory.

METHODS

A questionnaire sent to graduates of the program during the previous six years was judged the best means of securing the information needed to fulfill the objectives of the study. This particular period of time was chosen because it gave a group large enough to provide quantitative data, it was recent enough so that graduates should have been able to remember details with accuracy, and within this period few changes were made in the program, so that graduates' experiences were uniform. Specific items to be included in the questionnaire were determind by the investigator's observation of the program, consultation with instructors concerning their teaching objectives and criteria for evaluation, and interviews with current students for identification of factors seeming important to them. Most questions required only a check for response, which facilitated answering and tabulating of results. The final form was prepared after the preliminary draft had been criticized by medical technologists not included in the sample. It was then sent to participating graduates, with one follow-up letter to those who failed to respond the first time. Of the 169 questionnaires sent out, only six did not reach the addresses, and 114 responded. This constituted 70 per cent of the number who actually received the questionnaire. This response compares very favorably with similar studies, the usual yield being less than 60 per cent according to Rothney and Mooren (10). Results were probably not seriously affected by the response being less than 100 per cent, since a comparison of the responding group to the total group in respect to factors most apt to cause skewing (10) showed very little difference.

RESULTS

Professional Experiences of Graduates

The graduates had all held at least one position after graduation, with a total of 202 positions being held by the 114 respondents. Seventy-five per cent were currently employed, including over one half of the married women. Table I summarizes the types of positions held by graduates, in terms of institutions, size of laboratory, kinds of work performed by the technologist.

Satisfaction with their present positions was expressed by two thirds of the group, and only 7 per cent were thoroughly dissatisfied. The most frequently checked reasons for dissatisfaction were low salaries and lack of variety in work. The most frequent volunteered comments were unsatisfactory relationships with pathologists and staff, poor work being done in the laboratory and unqualified fellow workers, and lack of professional recognition. (See Table II). These volunteered comments were probably more important than the number would indicate, since they were not suggested by the wording of the questionnaire but rather spontaneous replies.

Meeting the Educational Objectives

General Effectiveness

Graduates felt well satisfied in general with the training they received, as indicated by the responses to questions along this line. Seventy-five

per cent evaluated the whole program as "excellent," 91 per cent felt that it gave good preparation for the Registry examination, 94 per cent good preparation for work in other laboratories.

TABLE I

Professional Experience of Graduates—An Analysis of the Total Number of Positions Held

TYPE OF LABORATORY*

Small General	Large Departmentalized	Research
40%	47%	13%

TYPE OF INSTITUTION†

University Hospital	Other Hospital Laboratories	Non-Hospital Laboratories
28%	45%	27%

TYPE OF WORK PERFORMED‡

General Varied	General, Rotating	Specialized
31%	19%	50%

PERCENTAGE OF TIME IN DIFFERENT LABORATORY SPECIALTIES‡

Hematology	37%
Chemistry	30%
Bacteriology	13%
Blood Bank	13%
Tissue Technique	7%

PERCENTAGE OF POSITIONS IN EACH SPECIALTY§

Hematology	33%
Chemistry	27%
Blood Bank	17%
Tissue Technique	12%
Bacteriology	11%

* based on 202 positions.
† based on 178 positions.
‡ based on 169 responses.
§ based on the 89 positions designed as "specialized".

Attitudes.

In common with most service professions, the development of certain attitudes is an important aspect of competency, and the acquisition of

TABLE II
Reasons Given by Graduates for Dissatisfaction with Positions

Response	Number Checking Response
Listed Items	
Salary too low	41
Work too routine	20
Atmosphere in the laboratory poor, inadaquate supervision	18
Too much overtime or call duty	12
Not enough responsibility	10
Not enough patient contact	8
Type of technical work assigned	7
Too much responsibility	3
Dissatisfaction with Medical Technology	2
Volunteered Comments	
Unsatisfactory relationship with pathologist and staff	6
Working with unqualified personnel	5
Lack of professional recognition	5
Poor quality of work done in the laboratory	4
Inadequate laboratory facilities	3
Hours scheduled to work	2

these is therefore a valid objective of the educational program. The two which seemed most amenable to evaluation were professional interest on the part of former students, and recognition of the necessity to continue their education following graduation, at least informally. One indication of professional interest would be the importance ascribed to the certifying examination. Ninety-six per cent took the examination, and 97 per cent felt it was an advisable requirement of employment. But in respect to their own organization much less concern was indicated, with only one fourth belonging to even a local chapter of the ASMT (American Society of Medical Technologists). In general, medical technologists were satisfied with their choice of vocation, with over two thirds enthusiastic and only 6 per cent disappointed.

One favorable indication that graduates recognized the necessity to continue study in their field was found in the 4 per cent who already had earned advanced degrees, the 10 per cent who had definite plans to begin, and 27 per cent who were considering it for some time in the future.

Skills and Knowledge

The instructors also had as objectives the acquisition of specific skills and knowledge which were considered essential to good performance in medical technology. Figure 1 shows the ratings given to the "problem-solving skills." Those having to do with theoretical knowledge were rated "superior" by more than one half the graduates, and the practical competencies were in the range below 50 per cent, with nearly one fifth rating the "ability to evaluate and compare procedures" as definitely "inadequate."

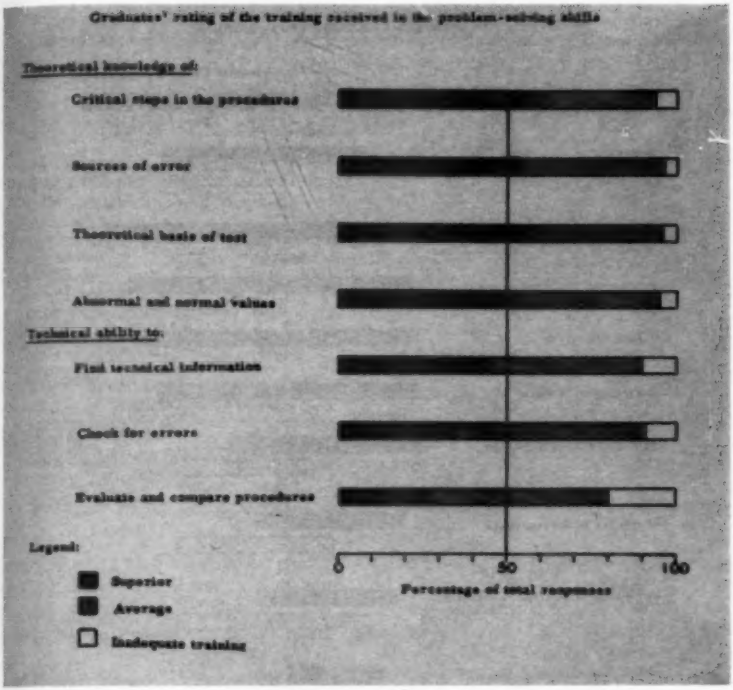

Figure 1

Other skills considered important were studied in relation to the degree of competency achieved and the importance ascribed by the working technologists. The final evaluation of adequacy was a composite of these two values so that "superior" ratings would only be considered necessary if the particular skill were "essential" to the respondent. When this was done, there were three groupings of rating for adequacy of training related to its importance. The highest with more than 70 per cent giving "adequate" ratings, the middle with between 47 and 60 per cent giving "adequate" ratings to these skills, and the lower group with the majority indicating training had been inadequate. In this last classification were "administrative procedures" and the "preparation of reagents." In general the program had the proper emphasis, with students rating "superior" those competencies regarded as "essential," and those which had lower ratings were usually not essential, with the exceptions noted above. (See Figure 2 for complete tabulation of these results.)

Some criteria to serve as indicators of the quality of training in their specific departments were also suggested by instructors. Results of these are given in Table III. These cannot be used to make comparisons be-

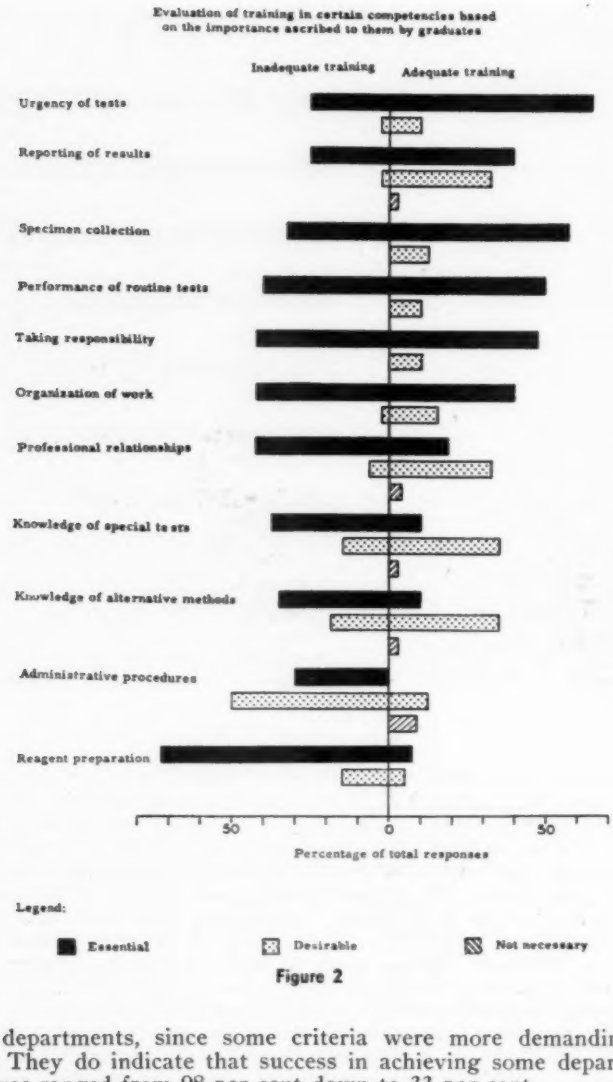

Figure 2

tween departments, since some criteria were more demanding than others. They do indicate that success in achieving some departmental objectives ranged from 98 per cent down to 33 per cent.

Specific suggestions of additional material were made in comments throughout the questionnaire. In hematology the greatest emphasis was placed on more experience in abnormal blood and bone marrow smears. In bacteriology the main need expressed was for more practice in rou-

TABLE III
Special Skills Acquired with Reference to Individual Departments in the Laboratory

	Percentage of Those Responding		
	Acquired	Doubtful	Not Acquired
Blood Banking			
The importance of blood banking procedures.	98	..	2
Hematology			
Performance of normal differentials and the recongnition of abnormal ones.....	79	6	15
Bacteriology			
Difference between clinical and scientific bacteriology.....	67	..	33
Chemistry			
Ability to standarize photometric procedures.....	67	5	28
Histologic Technique			
Ability to cut, stain, and mount routine paraffin sections.....	64	10	26
Bacteriology			
Ability to perform and interpret routine cultures.....	33	4	61

tine cultures and in related specialties of parasitology, serology and mycology. More emphasis on special and alternative procedures were suggested for clinical chemistry. In histologic technique students desired more materials for practice and more opportunity for special staining.

Effectiveness of Teaching Methods Used

Of special interest to instructors were the relative effectiveness of specific methods and program organization. Graduates were asked to rate the value of these items, listed in Table IV. From the table it can be seen that most methods were very useful in helping students learn the procedures, since nearly all were given the highest rating by more than two thirds.

The three ways of giving laboratory experience, a separate student laboratory, routine clinical laboratory, and by week-end work, were all rated as valuable with the last two having higher ratings. In another question 60 per cent answered that the student laboratory had a definite role as part of the teaching program and should definitely not be discontinued. Comments corraborated the importance of week-end and night work as preparation for future responsibility.

Specific devices used as adjuncts to laboratory instruction were also considered effective by most. Check-off requirements, a specific number of determinations to be performed for each procedure before working on patient specimens, were rated as "valuable" by 80 per cent. Even more gave this rating to the demonstrations of new or special procedures which were presented to the class by laboratory instructors before the

TABLE IV
Rating Given by Graduates to the Relative Usefulness of Specific Methods and Teaching Techniques

Techniques Used	Average of All Departments
	Percentage Rating Item "Valuable"
Laboratory Experience	
Student	58
Clinical	76
Work	75
Special Techniques	
Check-offs	80
Notebooks	
For learning	65
For reference	64
Practical examinations	75
Demonstrations	83
Academic Work	
Library reference	29
Lectures, theory	78
Correlation, lectures and laboratory	74
Seminar	31

actual performance of the tests by students. Notebooks were considered equally as important for future reference as for a means of learning the material, being rated "valuable" by 65 per cent in each case.

In the academic aspects of the program, lectures given by the Department of Pathology were rated "valuable" by 78 per cent. But library assignments were so considered by less than 30 per cent. The student seminar was the most controversial item in the list, eliciting much unsolicited comment and opinion being almost equally divided between the three ratings of "valuable, of some value, of little value."

One of the aspects of instruction found to be important was the amount and kind of requirements demanded of students in terms of individual responsibility and of work assignments. Two thirds felt that both were sufficiently well-balanced with sufficient amount for adequate learning without too heavy demands. Of the thirty-five per cent who criticized this aspect in any department, 31 per cent did so on the side of demands being insufficient rather than too heavy. Comments all spoke in favor of higher, not lower, standards.

DISCUSSION

This follow-up study has provided specific information which could be useful to laboratory supervisors and instructors in remedying some of the disadvantages of medical technology as a profession and in improving training programs. Since the scarcity of trained technologists is becoming an acute problem, the attempt to eliminate the major dissatisfactions might provide additional incentives for them to stay in the field and for successful recruitment of students to take up this area of

study. It is encouraging that the criticisms expressed are often peripheral to the vocation, such as working conditions and staff relations rather than inherent in the nature of the work itself. Thus they are more amenable to improvement.

The high degree of satisfaction with their training should be encouraging to those directly concerned with students' instruction. However, the study also has specific implications for organization and instruction in medical technology programs by revealing needs of technologists and the strengths and weaknesses of the program in meeting them. For example, the desire expressed by technologists for more professional recognition and yet the small amount of interest developed during training in the ASMT (American Society of Medical Technologists) points up the need for more planned interpretation by technologist instructors.

In respect to the evaluation of training as related to specific skills and knowledge certain emphases are suggested. Since students reported better background in theoretical aspects of problem-solving skills, more practical experience might be provided by setting up problems for students to solve after the routine instruction in a department had been completed. Or they might make comparisons individually of various procedures and report the results to the class. Also revealed by the study was the necessity for providing more practical experience in reagent preparation. Although emphasis on this aspect is important in all training programs, it is especially so in those laboratories which have access to use of a reagent laboratory and therefore have no adequate facilities or stocks of chemicals. Administrative procedures, such as organization of work, supervision of personnel, and ordering supplies, although requested by former students, are not usually taught when in training but "on the job" after some practical working experience. Probably graduates had been given supervisory responsibilities prematurely and thus felt the need of training in this aspect. With the need for training in so many other procedures, which are constantly increasing, it would seem this is not practical in a student program.

The volunteered suggestions of additional material varied in the departments. In hematology and chemistry respondents would have liked instruction in a wider variety of determinations of a special nature, while in bacteriology and histology technique they felt they needed more opportunity for practice in routine procedures. They reported that it was expected that they be acquainted with the more advanced chemical procedures, especially having trained in a university program, and with abnormal blood smears, in those hospitals where expert medical consultation in this field was not available.

In the consideration of special teaching devices, nearly all were rated very high, indicating that most special methods of instruction will be helpful to at least part of the class. The usefulness of the notebooks, especially for future reference, was unexpected to instructors, and indicates the desirability of continuing their use. The very high value ascribed to laboratory demonstrations suggests that this method might be used even more extensively, especially in acquainting students with a variety of procedures for which a familiarity is desirable but which cannot be practiced because of lack of time or facilities. The individual de-

partments whose demonstrations receive the highest ratings planned and carried these out very carefully.

Even though the student laboratory was rated as less important for learning, graduates nevertheless did not want to discontinue them in favor of complete instruction being carried out in the clinical laboratories. Many felt there was a real value in having the opportunity to learn a procedure thoroughly before working in the clinical laboratory where they felt they were interfering with the regular work. The importance ascribed to the week-end work in developing responsibility and initiative indicates that students should be given this opportunity whenever possible, especially in university programs where student laboratories are used.

The low ratings of the student seminar seems to have been because of the poor oral presentation made by the students to the class. Most felt that they had learned a great deal in the preparation of their own reports. Library assignments received a poor rating also, apparently because of infrequent use rather than ineffectiveness in the method per se. This suggests that these both have value if properly and sufficiently used. Students might be coached in presentation as well as preparation of reports. One successful experiment observed by the investigator consisted of assigning a small group of medical technology students to graduate students or working technologists in the department, the role of the latter being to aid the undergraduates in finding, preparing, and presenting the material. This resulted in marked improvement in presentation and enthusiasm.

The fact that one third of the students criticized courses for insufficient demands in responsibility and work indicates that students learn more from courses with high standards and do appreciate them, at least in retrospect.

This study was also of value to the instructors who participated in it, as it made them more aware of their objectives, and the possibilities for improvement even in an effective program. It also provided a model by which a future study might be carried out.

SUMMARY

A follow-up study of the graduates of a medical technology program and the results are described. Findings of this study gave useful information in respect to graduates' professional experiences, to an evaluation of their professional training, and to their rating of the relative effectiveness of specific instructional methods. These have implications for recruitment of technologists, as well as for the development and improvement of medical technology teaching programs.

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ABSTRACTS

NONSPHEROCYTIC CONGENITAL HEMOLYTIC ANEMIA

The great majority of cases of hereditary hemolytic disease fall into 1 of 4 groups: hereditary spherocytosis, thalassemia, sickle cell disease (and allied conditions associated with abnormal hemoglobins) and hereditary elliptocytosis with hemolytic anemia. However, in recent years a number of cases of congenital hemolytic disease which differ from the above disorders has been reported.

In the present series, the red corpuscles were of normal shape, of normal or slightly increased diameter and were moderately hypochromic. A proportion appeared as target cells (occasionally to 2%). Basiphil stippling was present in 1-5% of the red corpuscles. In all cases there was a small proportion of red corpuscles which were smaller than normal and which stained somewhat more deeply than normal; the margin of some of these was irregular, giving the appearance of irregularly contracted corpuscles. Reticulocyte counts were moderately increased, ranging from 8-14.4%.

The osmotic fragility of fresh blood was either slightly decreased (3 cases) or normal (1 case). The osmotic fragility of incubated blood was determined, showing much greater resistance to hemolysis than did normal incubated blood. Mechanical fragility was performed in 2 cases and found to be normal.

Autohemolysis was measured in 2 cases and found to be slightly increased. deGruchy, G. C., Santamaria, J. N., Parsons, I. C. and Crawford, H., Hematology Clinical Research Unit, St. Vincent's Hospital, Melbourne, Australia. *Blood*, 16, 4, 1960, pp. 1371-1397.

SPECTROPHOTOMETRIC DETERMINATION OF CATECHOLAMINES IN URINE*†

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Analysis of urine for catecholamines is a valuable diagnostic aid in detecting the presence of tumors of chromaffin tissue, the so-called pheochromocytoma (Sjoerdsma, 1959). The usefulness of such a test was first reported in 1950 by Engel and Euler of Sweden. The presence in normal human urine of pressor amines of catechol type had been reported by Holtz and coworkers in 1942 and 1947. Euler and Hellner (1951) confirmed these results using biological methods which enabled them to differentiate between epinephrine and norepinephrine. Both groups of investigators found evidence for the presence of dopamine, a weakly active pressor amine, in normal urine.

Dopamine (hydroxytyramine or 3, 4-dihydroxyphenylethylamine) is the immediate precursor of norepinephrine *in vivo* (Goodall and Kirschner, 1957). It is present in urine in considerably larger amounts than either epinephrine or norepinephrine (Euler, Hamberg, and Hellner, 1951). In cases of pheochromocytoma the excretion of this catecholamine as well as the epinephrines may be markedly increased (Euler, 1951; McMillan, 1957). Accordingly, the detection of pheochromocytoma should involve analysis for all three of these substances.

A new chemical method for quantitative determination of catecholamines has recently been developed in our laboratory.[14,16] Using this method under the conditions described herein, the sum of epinephrine, norepinephrine, and dopamine in a solution can readily be determined. Application to routine analysis of urine has been made possible by the development of a simple extraction procedure by which the catecholamines are extracted from urine in high yield and concentrated to a relatively small volume in one single process. This is accomplished by using a cation exchange resin in accordance with the well-known principles of ion exchange chromatography. (See Kunin, 1958.)

Reagents.

1. Amberlite CG-50, particle size 150 to 200 mesh. See Part I (a).
2. Sulfuric acid, 2 N, 1 N, 0.01 N.
3. Sodium hydroxide, 1 N.
4. Hydrochloric acid, 3 N.
5. Phosphate buffer, 0.4 M, pH 6.0.
6. Acetate buffer, 0.1 M, pH 5.0.
7. Iodine, 0.1 N in 0.1 M potassium iodide. Dissolve 12.7 gm iodine in about 150 ml of water containing 18 gm of potassium iodide and dilute to 1 L. Allow several hours for this to dissolve.
8. Sodium thiosulfate, 0.05 N in 0.002 M sodium carbonate. Dissolve 12.41 gm $Na_2S_2O_3 \cdot H_2O$ plus 50 mg Na_2CO_3 and dilute to 1 L with water.

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9. Sodium bisulfite ($NaHSO_3$). This is used in solid form.
10. DL-Norepinephrine hydrochloride standard solutions. See (e).

PART I. EXTRACTION METHOD

Theory. In common with amines in general, catecholamines can exist either as the "free base" (M) or as a salt (M•HA) formed by combining with a molecule of an acid (HA). The salts readily ionize to give a positively charged catecholamine molecule. (/MH^+). As a positively charged molecule the catecholamine can be readily adsorbed onto a cation exchange resin (Bergström and Hansson, 1951) by passage through a column of the resin under appropriate conditions. As such a resin, we have found Amberlite CG-50 to be most satisfactory. The pH range of effective operation of this resin is 5 to 14. Although its exchange capacity is greater at high pH values than at low, we have selected a low pH because catecholamines are stable only in an acid medium. For this reason, we use urine adjusted to pH 5.5 and pass it through a column of resin previously washed with phosphate buffer of pH 6. Separation of catecholamines from all urinary constituents which cannot be adsorbed onto the Amberlite, including the pigments of normal urine, is thus accomplished.

Elution of the catecholamines from the resin can be achieved by addition of mineral acid. Only small amounts of such acid are necessary since the resin, containing carboxylic acid groups, is of the weak acid type. Consequently, it is possible to obtain the desired amines from the column in a relatively small volume of eluate. There may be some turbidity in the extract due to fine particles of resin, but a correction for this is easily made as described in the analytical procedure below. A clear extract results if the resin has been regenerated several times before use.

Procedure. (a) **Preparation of Amberlite Column.** A suitable column can be made from an ordinary 50 ml burette or from glass tubing (11 mm internal diameter) to which a stopcock is attached. A small plug of glass wool is placed just above the stopcock to support the Amberlite. The stopcock must be properly greased and must be kept firmly in place, otherwise it may leak after the alkali has been passed through it. A wide bore (2 mm) stopcock is preferable. The column including the stopcock bore and tip is filled with water so that no air bubbles remain. After allowing a 3 gm portion of Amberlite CG-50 to swell in water for about 15 minutes, the resin is transferred to the column as a slurry. *The column should not be allowed to run dry.* A fluid level of at least 1 or 2 mm should be kept above the resin when the column is not in use. Using a drop rate equal to 1 to 2 ml per minute, the following reagents are added in succession, allowing each to drip through until the meniscus just disappears before adding the next:

35 ml N H_2SO_4
25 ml H_2O
35 ml N NaOH
25 ml H_2O
25 ml 0.4 M phosphate buffer, pH 6.0.

When the alkali is added the resin will swell slowly. Time should be allowed for maximum swelling (about one hour) before adding the phosphate. Otherwise the drop rate may be very slow at later stages. A slower drop rate is recommended when washing with NaOH. Using the above procedure the drop rate is usually self-adjusting to between 1 and 2 ml per minute, except at the alkali stage. After washing with phosphate buffer, the column is ready for passage of urine which has been previously adjusted to pH 5.5 as described below.

(b) **Preparation of the Urine.** It is necessary to have an accurately timed urine specimen, since the units of measurement recommended are micrograms of norepinephrine equivalents *per hour*. A time interval of at least 3 hours is recommended, although only 1.5 hours of urine are used per determination. It is helpful to analyze one portion (1.5 hours of urine) for unconjugated (free) catecholamines and another for conjugated plus unconjugated (total) catecholamines. To determine the latter, it is necessary to hydrolyze the urine.

For the hydrolysis procedure we have adjusted a 1.5 hour portion of filtered urine to pH 1.5 with 3 N HCl and immersed the flask containing this urine in a vigorously boiling water bath for 20 minutes, taking care that the level of urine in the flask is below the level of water in the bath. After 20 minutes the flask is immediately removed and immersed in an ice bath with stirring or swirling for rapid cooling.

In case the 1.5 hour urine volume is greater than 200 ml we prefer to use only 200 ml and calculate the time it represents in hours. This is designated "h" in the calculations.

The hydrolyzed and unhydrolyzed portions of filtered urine are then adjusted to pH 5.5 by careful dropwise addition of N sodium hydroxide with constant stirring, preferably with a plastic-coated magnetic stirrer. In this step it is necessary to guard against the pH going much above 6 since catecholamines are destroyed in an alkaline medium. Brom cresol purple is useful as an external indicator for following the pH adjustment. The final adjustment should in all cases be checked with a pH meter.

(c) **Adsorption of Catecholamines on Amberlite.** The filtered urine sample adjusted to pH 5.5 is added to the column of resin prepared as described under (a) and allowed to pass through at a rate of 1 to 2 ml per minute. An automatic setup for this step is easily arranged by using a long stem separatory funnel (500 ml) with the stem extending into the column for 5 or 6 inches. Having closed the stopcock of the separatory funnel, the urine is poured into the funnel followed by closing its top with a tightly fitting stopper, thus preventing air from entering the funnel from its top. The stopcock of the funnel is then opened cautiously and urine allowed to run onto the column. The flow of urine will stop automatically when the level of urine in the column is above the tip of the separatory funnel. With the top of the funnel tightly closed air can enter the funnel only by way of the stem and the opened stopcock when the level of urine in the column is such that the tip of the stem is no longer submerged. As air enters the separatory funnel, some urine will flow out thus automatically regulating the flow.

(d) **Washing the Column.** After all the urine has dripped through, the separatory funnel is removed and a 25 ml portion of water is added directly to the column and allowed to drip through in order to wash out all the unadsorbed material. All effluents up to this point are discarded.
(e) **Eluting the Catecholamines from the Column.** Elution is accomplished by adding to the column the following reagents in succession:

10 ml 2 N H_2SO_4
15 ml 0.01 N H_2SO_4

and collecting the eluate in a flask containing 0.5 ml N sulfuric acid. Analysis of the eluate is described in Part II.

(f) **Regenerating of Amberlite.** The column may be used over and over as long as the resin is regenerated by repeating the sequence of reagents as specified under (a). The amount of N sulfuric acid used in regeneration, however, can be reduced from 35 ml to 25 ml.

PART II. ANALYTICAL METHOD

Theory. Catcholamines extracted as above are oxidized at pH 5 to the corresponding aminochromes which in turn are converted to the bisulfite derivatives by reaction with sodium bisulfite. Light absorption in the ultraviolet region (λ max 360 mμ) is measured and compared with a standard norepinephrine solution or a norepinephrine calibration curve.

In the preliminary report of our method[16] sodium bisulfite was not added as such. At that writing the chemical nature of the catecholamine derivative having the characteristic intense absorption which we were measuring was not as yet established. A derivative of adrenochrome having the same ultraviolet absorption characteristics was reported independently by J. Van Espen[17,18] of Belgium. Formation of this derivative, postulated by this investigator to be a bisulfite addition compound, was accomplished by addition of sodium bisulfite to an aqueous solution of adrenochrome. R. A. Heacock (1959) has reviewed the evidence concerning the chemical constitution of this derivative and agreed with Van Espen that it is an addition type of compound rather than a reduction product.

In our method as originally described formation of the bisulfite derivative occurred slowly as the reaction mixture was allowed to stand for some 18 hours, during which time sodium bisulfite was apparently generated in the reaction mixture. By adding sodium bisulfite immediately following oxidation to the aminochrome stage, we found that the characteristic absorption peak is obtained much more rapidly. Consequently, we have modified[14] our original method to include the addition of sodium bisulfite. The spectrophotometric readings can then be made half an hour following the addition of the bisulfite, thus shortening considerably the time for completing the determination.

A different method for determining epinephrine as the bisulfite derivative of adrenochrome has been published independently by Van Espen.[19]
Procedure. (a) **Adjustment of pH of Eluate.** To facilitate this adjustment and to enhance the stability[17] of the aminochromes during the oxidation stage, enough sodium acetate is added to make the solution ap-

proximately 0.1 molar in acetate (approximately 400 mg of $NaOAc \cdot 3H_2O$, the average final volume of eluate being 30 ml). Then by dropwise addition of N sodium hydroxide with continuous stirring or shaking the pH is adjusted upward to 5.0. For stirring we recommend a plastic-covered magnetic stirrer. A piece of congo red paper in the solution will indicate when the pH is approaching 4. Brom cresol purple is helpful as an external indicator if external electrodes are not available for electrometric titration. In any event, the pH should be checked with a pH meter as it approaches 5.

The final volume (designated "v") of the solution adjusted to pH 5 is measured with a 50 ml graduated cylinder since it is necessary to know this volume for purposes of calculation. See (f).

(b) **Analytical Reaction.** To 2 ml portions of the above solution (eluate adjusted to pH 5) contained in a small flask suspended in a water bath at 30° C, is added 0.4 ml of 0.1 N iodine in 0.1 M potassium iodide. After exactly 15 minutes the oxidation is stopped by addition of 0.9 ml of 0.05 N sodium thiosulfate ($Na_2S_2O_3$) in 0.002 M sodium carbonate, with mixing. Excess iodine is thus removed. The resulting solution will be slightly pink if elevated amounts of catecholamines are present. Whether a pink color is seen or not, 3 to 6 mg of sodium bisulfite ($NaHSO_3$) is promptly added and the solution again thoroughly mixed. Standard solutions of norepinephrine in 0.1 M acetate buffer at pH 5 can similarly be analyzed for purposes of constructing a calibration curve. Fig. 1, see (e).

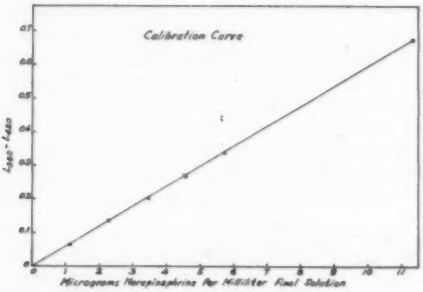

Fig. 1. Calibration curve for norepinephrine. See text for procedure.

(c) **Blanks.** A separate blank must be prepared for each eluate or standard solution as follows: to 2 ml of the eluate or standard solution is added 1.3 ml of water.

(d) **Determination of the Optical Density.** Optical densities of the final solution in (b) at 360 and 450 mμ are determined half an hour to one hour after the addition of sodium bisulfite or alternately at 360 and 420 mμ 18 to 24 hours after addition of the bisulfite. We have used a Beckman spectrophotometer, Model DU with 1 cm cuvettes. If the 360 mμ reading exceeds the 420 mμ or 450 mμ reading by as much as 0.045 units, it is

strongly recommended that the shape of the absorption curve between 300 and 420 mμ be determined. If an atypical shape is found for this curve it is well to inquire whether the individual has been given any medication that may have produced interference in the test. Such interference has been found for phenyl-azo-diamino-pyridine ("Pyridium") and hydroxychloroquine ("Plaquenil"). Typical absorption curves from two normal individuals and one case of pheochromocytoma are shown in Fig. 2. The top curve of each pair is for the hydrolyzed urine and represents conjugated plus unconjugated catecholamines while the bottom

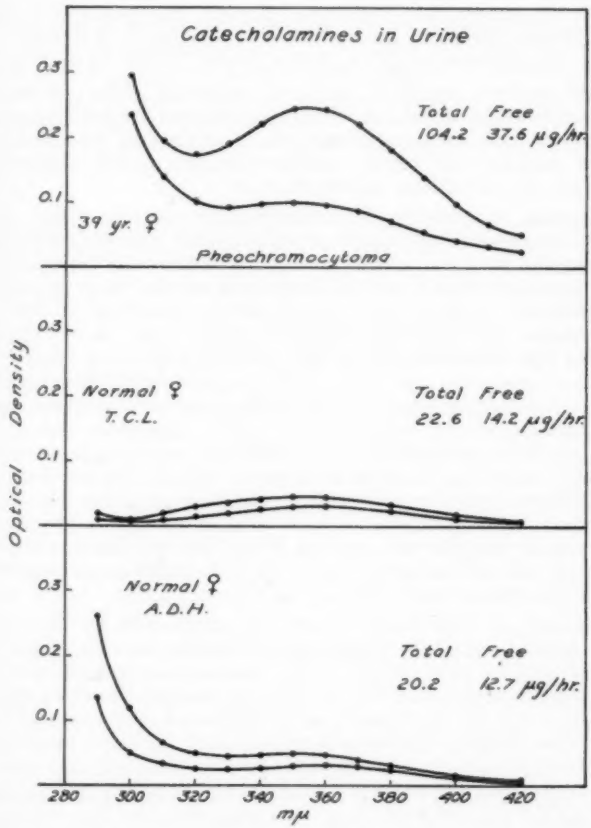

Fig. 2. Absorption curves obtained using 1.5 hours of urine in the analysis as described in text. Upper curve in each pair represents total catecholamines, lower curve represents free catecholamines.

curve is for the unhydrolyzed urine and represents unconjugated catecholamines alone. The portion of the curve at wave lengths below 320 mμ can be quite variable. This variability is attributed to small differences in the amount of sodium bisulfite added in the analysis and does not significantly affect the results. The height of the curve at 360 mμ and especially the differences between the 360 mμ and 420 mμ readings are, however, of greatest importance. The optical density reading at 420)or 450) mμ is due in part to the small amount of background absorption contributed by the reagents. Since these reagents contribute essentially the same amount to the optical density reading at 360 as at 420 mμ, an appropriate correction for the background absorption at 360 is obtained by subtracting the 420 mμ reading from the 360 mμ reading. This subtraction will also correct for small differences in turbidity, if any, between the sample and its blank. A further purpose served by this subtraction is explained under "Calibration."

As mentioned previously, a perfectly clear eluate is not required in this method since an individual blank is prepared for each eluate. Small differences in cloudiness between the sample and its blank can be caused by adding too much sodium bisulfite. Such differences are corrected for by the above subtraction.

(e) **Calibration.** A norepinephrine calibration curve is shown in Fig. 1. Within the concentration limits of the graph it is essentially a straight line represented by the equation, $x = 16.5\ y$, where x is the concentration of norepinephrine (free base) as expressed in the final solution and y is the difference between the optical density readings at 360 mμ and 420 mμ. Linear relationships between x and y are also obtained with epinephrine and dopamine. Using the conditions specified herein, straight lines of very nearly the same slopes are obtained with all three catecholamines. The determination can be made more sensitive for the latter two catecholamines by reading at slightly different wave lengths, however, we have elected to use conditions which give as closely as possible the same results on a microgram for microgram basis for all three catecholamines. In this way an accurate measure of the sum of these compounds is obtained. The additional reason referred to above for subtracting the 420 mμ reading from the 360 mμ reading is that by so doing the calibration curves for the three catecholamines are within 8.5 per cent of each other.

Construction of a calibration curve is illustrated by the following. A sample of 4.54 mg of DL-norepinephrine hydrochloride (equivalent to $4.54 \times 169.2/205.7$ or 3.74 mg of free base) was dissolved in 200 ml of 0.1 M acetate-acetic acid buffer, pH 5.0. Further dilutions were made by diluting 5, 10, 15, 20, and 25 ml portions of this solution to 50 ml with the same buffer. The resulting standard solutions were analyzed by the routine procedure as described under (b) and (c). Optical density readings at 360 and 420 mμ were made some 20 hours later. The differences in optical densities at these two wave lengths ($D = L_{360} - L_{420}$) were then plotted against concentration expressed in micrograms of free base per ml of final solution. In the calculations it is necessary to take into account the fact that further dilution of each standard solution

is made during the analytical procedure; i.e., 2.0 ml to 3.3 ml. As an example, the concentration of the highest standard is calculated as follows: $4.54 \times \frac{169.2}{205.7} \times \frac{2.0}{3.3} \times 1000 = 11.32$ microgram per ml of final solution. Such a calibration should be performed periodically as a check on technique and reagents.

(f) **Calculations.** The results of an analysis can be calculated using the following formula:

$$C = D \times 16.5 \times \frac{3.3}{2.0} \times \frac{v}{h} = 27.2 \times D \times \frac{v}{h}$$

where

C = micrograms of catecholamines per hour expressed as norepinephrine equivalents;
D = $L_{360} - L_{450}$ (½ to 1 hour after bisulfite)
or $L_{360} - L_{420}$ (18 to 24 hours after bisulfite);
v = final volume of eluate in ml;
h = time interval, in hours, representing the volume of urine used in analysis.

RESULTS

Analyses of urinary catecholamine levels have been made on 103 different individuals: 45 normal controls and 58 patients. Many of these individuals have been analyzed several times for purposes of studying the per cent recovery of added catecholamines or for elucidating the effect of various factors on catecholamine excretion levels. In the present report we are concerned primarily with recovery studies and with establishing normal ranges.

Recovery Studies. Typical results of recovery studies are shown in Table I. A known amount of a given catecholamine (the amount expressed in terms of micrograms of norepinephrine equivalents) is added to urine the catecholamine content of which has been previously determined. Analysis of the resulting urine is then performed and the micrograms of catecholamines found is compared with the amount calculated to be present. The recoveries of epinephrine and norepinephrine have been uniformly above 80 per cent. For dopamine the recovery tends to be somewhat lower, but it is nevertheless quite satisfactory, being consistently above 70 per cent.

Normal Ranges. For normal ambulating males we have found a range of 9 to 22 micrograms per hour. This is based on the results of a series of 15 normal males varying in age from 14 to 62 years (Fig. 4). Similarly, by analysis of a series of 30 normal ambulating females varying in age from 22 to 73 years, we have found a range of 8 to 18 micrograms per hour (Fig. 6).

Pheochromocytoma Range. According to the investigations of Euler and Strom (1957) among others, it seems well to regard any value for free catecholamines in the vicinity of three times the normal value as highly suggestive of pheochromocytoma. Since the mean (Fig. 6) for normal females is 12.69, we have regarded as highly suggestive of

TABLE I

Recovery Studies

Urine Used* a	Catecholamines as Norepinephrine Equivalents: Originally present*** b	Added† c	Total d	Found e	Recovery 100 e / d f††
		Epinephrine			
ml	μg	μg	μg	μg	μg
83**.....	15.2	10.0	25.2	24.1	95.6
83**.....	15.2	20.0	35.2	33.4	94.8
103**.....	17.1	18.55	35.65	33.2	93.1
103**.....	17.1	37.1	54.2	49.7	91.7
		Norepinephrine			
136**.....	23.3	6.6	29.9	28.9	96.6
136**.....	23.3	13.2	36.5	34.6	94.8
43**.....	20.0	6.6	26.6	26.0	97.8
43**.....	20.0	13.2	33.2	32.0	96.3
123.....	19.8	100.0	119.8	109.0	91.0
123.....	19.8	200.0	219.8	193.0	87.8
		Dopamine			
147**.....	13.3	5.15	18.45	16.6	89.9
147**.....	13.3	10.3	23.6	21.4	90.6
147**.....	13.3	15.5	28.8	23.7	82.2
147**.....	13.3	20.6	33.9	29.2	86.2
178.....	26.0	43.8	69.8	54.0	77.4
178.....	26.0	65.6	91.6	68.2	74.5

* Represents 1.5 hour urine volume. ** Diluted to 200 ml.

*** In 1.5 hour urine volume.

† Epinephrine (E) and dopamine (D) are given in micrograms of norepinephrine equivalents (NorEq). To convert NorEq to E, multiply by 0.921. To convert NorEq to D, multiply by 0.946.

†† Note: Values in column "f" were calculated by multiplying value in column "e" by 100 and dividing by value in column "d,"—hence $f = \frac{100\ e}{d}$

pheochromocytoma any value which is approximately three times this mean, namely 38 micrograms per hour. Among the female patients in our series of 42, there were two having values for free catecholamines in this vicinity. One of these was later proven to have had pheochromocytoma by microscopic examination of the tumor removed. The free catecholamine value found for this patient pre-operatively was 37.6 and the total catecholamine value 104.2 micrograms per hour (Fig. 2). The other patient who had a high catecholamine excretion level (55.4 total, 41.3 free) unfortunately died before we could obtain a second urine specimen for confirmation of our results. At autopsy no evidence of an adrenal tumor was found. Extensive searching for tumor of other chromaffin tissue was not carried out.

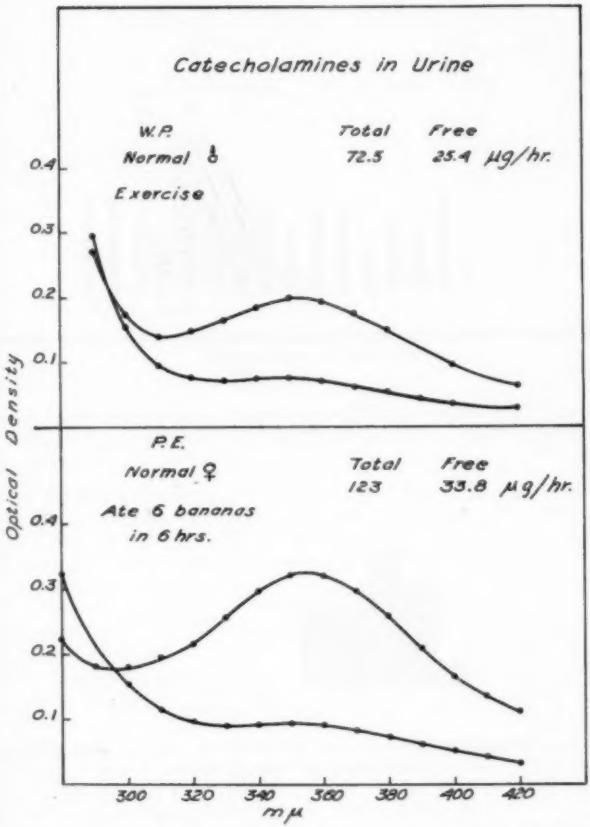

Fig. 3. Absorption curves obtained using 1.5 hours of urine in the analysis as described in text. Upper curve in each pair represents total catecholamines, lower curve represents free catecholamines.

The mean value (Fig. 4) for free catecholamines in the normal male series was 17.2. None of the male patients had excretion levels which approached the range suggestive of pheochromocytoma, namely three times 17.2 or 51 micrograms per hour.

Catecholamine excretion levels in pheochromocytoma have been reported[7] as high as 100 times the normal.

Effect of Exercise. While exercise may increase the excretion of both free and conjugated catecholamines, the increase in free catcholamines is much less than that in the conjugated fraction in normal individuals. The highest levels we have found in presumably normal individuals as

Fig. 4. Bar diagram showing free catecholamine values for series of normal ambulating male

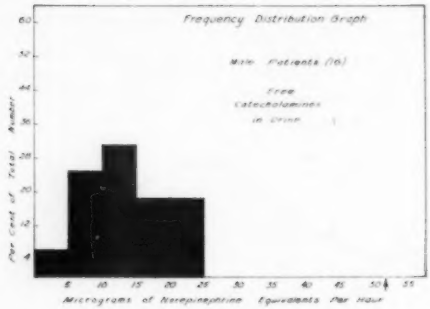

Fig. 5. Frequency distribution graph for free catecholamine values of male patients. The arr
indicates three times the mean.

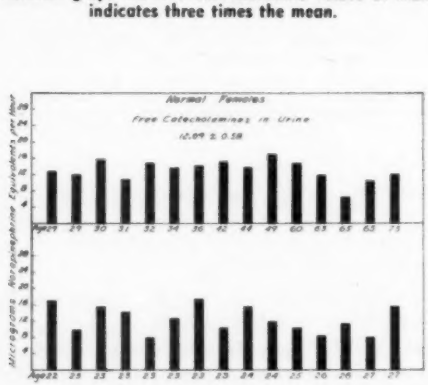

Fig. 6. Bar diagram showing free catecholamine values for series of normal ambulating femal

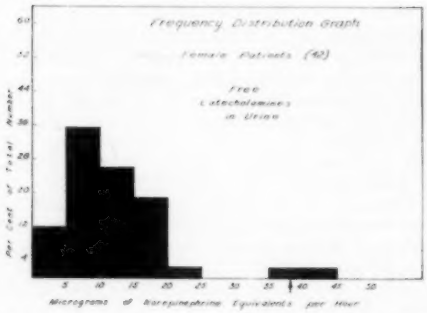

Fig. 7. Frequency distribution graph for free catecholamine values of female patients. The arrow indicates three times the mean.

a result of fairly vigorous exercise are shown in Fig. 3. These values while definitely elevated, still do not approach those obtained in pheochromocytoma.

Effect of Banana Eating. Interference in this test is caused by eating bananas (Fig. 3) because they contain fairly large amounts of catecholamines (Crout and Sjoerdsma, 1959). For this reason they must be excluded from the diet of patients undergoing this test.

SUMMARY

A sensitive, yet convenient and easily controlled, chemical method of analyzing urine for catecholamines is herein described in detail. Although this method is not as sensitive as the spectrophotofluorimetric methods currently in use, it has the pronounced advantage of being much more easily controlled than the fluorimetric methods.

The new method is based on extraction of catecholamines from urine by means of cation exchange resin and subsequent measurement of the ultraviolet absorption of the bisulfite derivative of the aminochromes which are formed on oxidation of catecholamines.

Normal ranges in terms of micrograms of norepinephrine equivalents per hour have been tentatively established for males and females as 9 to 22 and 8 to 18, respectively. These ranges are higher than the normal range established by methods which do not measure dopamine as well as the epinephrines. We believe it is important to include in the determine all three catecholamines, namely, dopamine, epinephrine, and norepinephrine, since the excretion of all three may be elevated in cases of pheochromocytoma.

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THE USE OF THE MUCICARMINE STAIN FOR A RAPID PRESUMPTIVE IDENTIFICATION OF CRYPTOCOCCUS FROM CULTURE*

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Cryptococcus (Torulosis, European blastomycosis) is a mycotic disease carried by the yeast *Cryptococcus neoformans* (Cryptococcus hominis, *Torula histolytica)*. The true incidence of this disease is difficult to assess, but many cases are undoubtedly undiagnosed or misdiagnosed.[5]

Cryptococcus neoformans is the only encapsulated yeast capable of invading the central nervous system of man. The demonstration of an encapsulated yeast in the cerebrospinal fluid is therefore prima facie evidence of Cryptococcus meningitis. However to rule out the possibility of artefacts, such as oil droplets, degenerated tissue cells etc., cultures are necessary.

In addition it has been pointed out, that not infrequently, patients with malignant lymphoma or leukemia may suffer from unsuspected coincidental widely diseminated cryptococcosis.[8]

Infection outside the central nervous system is not rare, although the exact incidence of occurrence of pulmonary or localized infections is also unknown. Demonstration of Cryptococcus neoformans culturally, from sputum or other areas may be difficult, particularly if this organism grows mixed together with Candida or other yeasts. During the first few days of growth or if the strain is poorly encapsulated, the colonies of Cryptococcus may be mistaken for saprophytic yeasts and discarded.

It must also be recognized that non-pathogenic Cryptococci, previously but erroneously called *C neoformans var innocuous*[1,4] may exist on normal skin.[7] The culture of an organism from any site in the body other than the central nervous system must be subjected to careful study for *C neoformans*. *C neoformans* can be differentiated from other non-myceliated yeasts by the following criteria[4]

1. Ability to grow at 37° C.
2. Ability to hydrolyze urea.
3. Capsule formation on Cryptococcus capsule agar.[6]
4. Virulence and capsule formation in mouse brain after intracebral inoculation.

Microscopic examination in india ink of colonies grown on Sabouraud's or blood agar reveals oval or round budding cells with capsules varying in size from a faint shell to 7 micra. No mycelia arthrospores chlamydospores or ascopores are formed.

In tissues, the organisms can be stained readily by the mucicarmine stain. (3) This stain is specific for mucopoly-saccharide in the capsule and cell wall of *C neoformans*. Since no other yeast pathogenic for humans possesses this clinical structure, the stain is considered specific for the organism.

We have recently had the occasion to study two cases of Cryptococosis in this institution, one of the nervous system, the other localized in the skin. In both cases, the organisms were poorly encapsulated and in the latter case it might possibly have been missed since the colonies at first resembled saprophytic yeasts. Proof of its identity as *C neoformans* required several

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weeks according to the criteria listed above. Rapid and accurate identification is even more important than ever in view of the reports of successful therapy with Amphotericin B.[2]

In view of the specifity of the mucicarmine stain for *C neoformans* in tissues and the fact that it stains the cell wall as well as the capsule, it was felt that perhaps the stain could be applied for the rapid presumptive identification of suspected colonies of Cryptococcus from culture plates. It is well recognized that further tests would be required to prove the identity of the organism, but if successful, the technique may hasten the identification and avoid missing it altogether. The procedure that was adopted was intended to be rapid, ample and utilize the commonly available mucicarmine stain.

METHOD

Nine strains of *C neoformans* were used in this study. All were isolated from human cases and fulfilled the criteria described above. Control organisms were from the type culture collection of the Bacteriology department of this institution. (Table I) All organisms were grown on Cryptococcus

TABLE I
Strains of Yeasts Used in Study

Organism	Strains	Capsule Size*
1. C neoformans	Roth	0
2. C neoformans	Rob	0
3. C neoformans	59-283	+
4. C neoformans	58-030	++
5. C neoformans	59-148	0
6. C neoformans	59-2347	++
7. C neoformans	56-55VA	+
8. C neoformans	Jackson	0
9. C neoformans	Fenr	0
Controls:		
Candida albicans (3 strains).		
Candida krusei.		
Saccharomyces cerevisiae.		
Torulopsis glabrata.		
Kloeckera brevis.		

* Abritarily measured on the basis of size in india ink mounts from colonies on Sabouraud's Agar, 5 days at 20° C.

capsule agar for five days at 37° C to increase their capsular size[6]* The organisms were then emulsified in water to make a thin smear, which we found to be important. The smears were air-dried and fixed in absolute methanol for 5 minutes. The slides along with the controls were air-dried again and placed in various dilutions of Mayer's mucicarmine stain† (an aqueous dilution was made instead of the alcoholic) for different periods of time, to determine the optimal procedures for Cryptococcus. After the staining, the slides were blotted dry and examined microscopically to determine at what time and what dilution the best results could be obtained.

† Media Incorporated, Newark, New Jersey.

The strains of Cryptococcus which were grown on capsule agar were also grown on Sabouraud's dextrose and Littman's agar at room temperature and at 37° C in order to simulate the usual growth conditions, in the clinical laboratory. No difference in the staining characteristics were noted in either case.

RESULTS

The dilution of mucicarmine stain usually used for tissue staining is 1:3. It was felt that perhaps the stain could be used undiluted for a short period of time. This was tried for, three, fifteen, thirty, and sixty minute periods. The capsules stained a faint pink shade but there was no staining of the cell wall. Dilutions of 1:1, 1:2, and 1:3 were then tried for the same periods of time (Table II). At fifteen minutes there was a slight staining of the cell

TABLE II
Results of Staining Cryptococci at Different Periods of Time with Mucicarmine Stain

DILUTIONS	STAINING TIME			
	3 Min.	15 Min.	30 Min.	60 Min.
Undiluted	0	0	0	0
1:1	0	+	+	+
1:2	0	++	++	++
1:3	+	+++	+++	+++

Controls gave a negative staining throughout.
Code:
0 Slight pink coloration of capsule but no staining of cell wall.
\+ Staining of capsule but no staining of cell wall.
++ Pale staining of capsule with a faint staining of cell wall.
+++ Dark staining of capsule with intense staining of cell wall.

wall in the dilution of 1:1. In a 1:3 dilution, the cell wall was stained an intense red with a dark staining of the capsule. This was decided on as the optimal timing and dilution for the Cryptococci. All of the controls gave a negative staining throughout.

It was found that if the organisms were packed too closely together they stained less intensely, hence it is important to make the smears thin.

SUMMARY

A rapid staining procedure using Mayer's mucicarmine stain for the presumptive identification of *C neoformans* from cultures is described. While further tests for proof of the identification of the organism would still be required, this simple stain may speed the identification and avoid missing this highly pathogenic organism on culture.

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